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Patentanmeldung Nr. Patent application No. Demande de brevet n°

99870046.2

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Blatt 2 der Bescheinigung
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Sesquiterpenoid synthase genes and their use for influencing bitterness and resistance in plants

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Title of the invention

Sesquiterpenoid synthase genes and their use for influencing bitterness and resistance in plants

Field of the invention

This invention relates to the use of sesquiterpenoid synthase genes, particularly genes encoding germacrene A synthase, to modulate (i.e. repress, induce or increase) the expression or activity of sesquiterpenoid synthases in plants, so as to influence sesquiterpenoid production of these plants. More particularly, the invention is directed to the inhibition of sesquiterpenoid synthase expression, reducing the production of sesquiterpene lactones in the plant. Particularly, in crops where these sesquiterpene lactones are associated with a bitter taste, such as chicory (*Cichorium intybus L.*), the invention can be used to obtain plants or plant parts that are less bitter. More particularly, this is achieved by reducing gene expression of germacrene A synthase by anti-sensing or co-suppression. Other aspects of this invention relate to the use of sesquiterpenoid synthase genes, more particularly genes encoding germacrene A synthase, to obtain increased resistance against insects, nematodes or micro-organisms in plants, to obtain increased formation of sesquiterpene lactones with attractive, e.g. medicinal, properties, and to obtain increased formation of germacrene A derived flavor and fragrance compounds or phytoalexins. This invention also relates to plant cells and plants transformed with one or more transgenes, which results in the modulation of activity of a sesquiterpenoid synthase therein.

All documents cited are incorporated herein by reference.

Background of the Invention

The sprouts of chicory (*Cichorium intybus L.*), known as the Belgian endive, are characterized by their slightly bitter taste, which is a limiting factor to its commercial value as food crop. The taproots of chicory, which are even more bitter, were used in former days as a coffee substitute. Because of their bitterness these roots are not very well suited for use as cattle feed and are mainly regarded as a waste product of chicory cultivation. Chicory roots of specific varieties have been demonstrated to be an interesting source for inulin and/or high-fructose syrup for which special extraction procedures have been developed (Perschak and Wolfslehner, *Zuckerind.* **115**(6):466-470, 1990); nevertheless, production of fructose from chicory roots requires the removal of the bitter taste in the course of the extraction procedure.

The bitter constituents of chicory and other vegetables have been associated with sesquiterpene lactones, more particularly the guianolides lactucin, 8-deoxylactucin and lactupicrin (van Beek et al. *J. Agric. Food Chem.* **38**: 1035-1038, 1990; Price et al. *J. Sci. Food Agric.* **53**: 185-192, 1990). Other sesquiterpene lactones identified in chicory are eudesmanolides, and germacranolides (Seto et al., *Chem. Pharm. Bull.* **36**:2423-2429,1988). The sesquiterpenoids belong to a very large family of plant products, the terpenoids, which have been associated with a variety of biological functions mainly related to plant-plant, plant-insect and plant-pathogen interactions. The production of terpenoids is based on a common biosynthetic pathway after which specific enzymes or synthases lead to the individual terpenoid structures.

The initial step of this pathway involves the fusion of three molecules of acetyl CoA to produce the C6 compound 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA). HMG-CoA is reduced to mevalonate by HMG-reductase, and mevalonate is in turn phosphorylated by two kinases, mevalonate- and phosphomevalonate kinase, to form 5-pyrophosphomevalonate. Pyrophosphomevalonate decarboxylase then converts the latter into isopentenyl pyrophosphate (IPP), which represents the first "active" isoprene unit or building block. Alternatively, IPP can be produced via the newly discovered mevalonate-independent deoxyxylulose pathway (Rohmer, In

Comp. Nat. Prod. Chem. Vol. 2, ed. by D. Cane, Pergamon, 1999). After isomerization of IPP to dimethylallyl pyrophosphate (DMAPP), prenyl pyrophosphate co-substrates are added to this basic unit to form longer chains. Two diphosphorylated building blocks generate geranyl diphosphate (GDP), a linear C10 intermediate, that can be converted into linear or cyclic products representing the monoterpenes. Addition of a third unit of IPP generates farnesyl diphosphate (FDP) from which the sesquiterpenoids are derived. The addition of one more IPP unit generates geranylgeranyl diphosphate (GGDP), the precursor for diterpenes, carotenoids, etc. The conversion of GDP, FDP and GGDP into terpenoid products is realized by monoterpene, sesquiterpene and diterpene synthases respectively, which give rise to stable end products or substrates for other enzymes catalyzing secondary transformations. It is assumed that the sesquiterpene lactones originate from a germacrane precursor that is formed from FDP by a germacrane synthase. The germacrane precursor is further cyclized to either a guiane skeleton (of the guianolides) or a eudesmane skeleton (of the eudesmanolides). This germacrane precursor has recently been identified as (+)-germacrene A (De Kraker et al *Plant Physiol.* **117:** 1381-1392, 1998).

Other vegetables, such as lettuce (*Lactuca salva and L. sativa*), radicchio (*Cichorium intybus*), endive (*Cichorium endivia*), and artichoke (*Cynara scolymus*) have also been demonstrated to contain sesquiterpene lactones as bitter constituents (Price et al., 1990, above; Herrmann K., *Z. Lebensm. Unters. Forsch.* **167**:262-273, 1967).

Examples of sesquiterpenoids associated with bitter taste are cnicin (from *Cnicus benedictus*), absinth (from the wormwood, *Artemisia absintha L.*), alantolactone and isoalantolactone (from *Inula helenium* roots) and helenalin (from sneezeweed, *Helenium autumnale*) (Fischer N., *Methods in Plant Biochemistry* **7**:187-211, 1991).

Several sesquiterpenoids have been described to have an anti-feedant activity on herbivorous insects and vertebrate herbivores. Examples of these are tenulin (from *Helenium amarum*; Arnason et al., *Journal of Natural products*, **50(4)**: 690-695, 1987) helenalin (from sneezeweed, *Helenium autumnale*), parthenin (from *Parthenium histerophorus*) (Picman A. and

Picman J., *Biochemical systematics and Ecology*, **12(1)**: 89-93, 1984) and linifolin A (Nawrot et al., *Prace Naukowe/OR*, **24**: 27, 1982). Many sesquiterpene lactones have been shown to possess pharmacological [parthenolide from feverfew (*Tanacetum parthenium*) has an anti-migraine effect (Hewlett et al., *Journal of the Chemical Society Perkin Transactions 1*, **16**: 1979-1986, 1996)], as well as anti-fungal, anti-bacterial, anti-protozoan, schistomicidal and molluscidal activities (Picman, *Biochemical Systematics and Ecology* **14(3):** 255-281, 1978). (-)-Germacrene A has been identified as the alarm pheromone in aphids (Bowers et al., *Science* **196**:680-681, 1976). Also, germacrene A has been postulated to be an intermediate in the formation of the important flavor compound nootkatone (Croteau and Karp, In *Perfumes: art, science and technology*, ed. by P.M. Müller and D. Lamparsky, Elsevier Science Publishers LTD, England, 1991), as well as an (enzyme-bound) intermediate in the biosynthesis of phytoalexins such as aristolochene, 5-epi-aristolochene, capsidiol, debneyol, and vetispiradiene (Back and Chappell, *J. Biol. Chem.* **270(13):** 7375-7381, 1995; Whitehead et al., *Phytochemistry* **28(3):** 775-779, 1989).

The biological structure and known functions of a large number of sesquiterpenoid lactones as well as the different methods by which they can be isolated is described in the review by Fischer (*Methods in Plant Biochemistry* **7**:187-211, 1991).
McGarvey and Croteau (*The Plant Cell* **7**:1015-1026, 1995) give an overview of the biosynthetic pathways of terpenoids and their regulation.
Chappel (*Annu. Rev. Plant Physiol. Plant Mol. Biol.*, **46**:521-47, 1995) reviews the biochemistry and molecular biology of the isoprenoid biosynthetic pathway in plants. Molecular comparison of a monoterpene, a sesquiterpene and a diterpene synthase demonstrates a strong similarity in gene-organization and in amino acid sequence within domains.

A number of genes involved in sesquiterpenoid biosynthesis have been isolated, of which some examples are given:
Two independent cDNA clones encoding 5-epi-aristolochene synthase (EAS) from tobacco have been isolated and characterized by Facchini and Chappell (*Proc Natl Acad. Sci. USA*, **89**:11088-11092, 1992). The cDNA encoding farnesyl diphosphate synthase was cloned and analyzed for *Arabidopsis*

thaliana by Delourme et al. (*Plant Molec. Biol.*, **26**:1867-1873, 1994) and for *Artemisia annua* by Matsushita et al. (*Gene*, **172**:207-209, 1996). Back and Chappell described the cloning and bacterial expression of vetispiradiene synthase found in *Hyoscyamus muticus* (1995, above). Molecular comparison of this sequence with that of tobacco EAS displayed identical intron-exon organization of the gene and strong sequence similarities, which is suggested to be reflective of the conservation of several partial reactions common to both enzymes (Back and Chapell, *Proc. Natl. Acad. Sci. USA*, **93**:6841-6845, 1996).

WO 9715584 describes the use of S-linalool synthase, an acyclic monoterpene synthase, in the genetic engineering of scent production.

The use of the limonene (monoterpene) cyclase in the control of corn rootworm, by inserting a nucleotide sequence coding for limonene cyclase into the plants is described in WO 9637102.

The inactivation of endogenous genes using either sense or anti-sense transgene constructs, has been demonstrated to be successful (Mol, J.N.M. et al., In *Homologous recombination and gene silencing in plants*, ed. by J. Paszkowski, Kluwer Academic Publishers, the Netherlands, 1994; Hamilton, A.J. et al., *Current Topics in Microbiology and Immunology* **197**, 77-89, 1995; Bourque J., *Plant Science*, 105:125-149, 1995; Cannon M. et al., *Plant Molecular Biology* **15**:39-47, 1990; Smith C.J.S. et al., *Molecular and General Genetics*, **224**:477-481, 1990). Also the inactivation of two non-homologous endogenous genes using a single sense gene construct has been reported (Seymour et al., *Plant Molecular Biology* **23**:1-9, 1993). The inactivation of an endogenous gene using constructs encoding ribozymes targeting endogenous genes is described by Haselhoff and Gerlach (*Nature* **334**:585-591, 1988) and in WO 89/05852.

Alternative methods have been described for decreasing endogenous gene expression. For instance, direct modulation of the endogenous gene using the chimeric RNA-DNA oligonucleotide technology. The method is based on the construction of a chimeric RNA-DNA oligonucleotide in duplex conformation with double hairpin caps on the ends, of which the sequence is

designed so as to align with the sequence wherein a mutation is targeted and so as to contain the desired nucleotide change and subsequent introduction of the chimeric oligonucleotide in the cell. A detailed description of this method and its efficiency for bringing mutations into endogenous genes is described by Cole-Strauss et al. (*Science*, **273**:1386-1389, 1996) and in patent n° US patent number 5,565,350.

Suppression of endogenous gene activity can also be achieved by introducing transgenes encoding inhibitors of the enzymatic gene product. Modulation of physiological functions using recombinant immunoglobulins is reviewed by Conrad and Fiedler (*Plant Mol. Biol.* **38**:101-109, 1998).

The aim of the present invention is to provide a polynucleic acid sequence encoding a sesquiterpenoid synthase, more particularly a germacrene A synthase.
Another aim of the present invention is to provide a process for producing a plant with modified sesquiterpenoid synthase activity.
Another aim of the present invention is to provide a process for producing a plant or plant parts with an increased content of germacrene A or sesquiterpene lactone metabolites thereof.
Another aim of the present invention is to provide a process for producing a plant or plant parts with reduced bitterness.
Another aim of the present invention is to provide a recombinant polynucleic acid encoding germacrene A synthase.
Another aim of the present invention is to provide a plant cell or plant, which is transformed with a recombinant polynucleic acid encoding a molecule having the biological activity of a germacrene A synthase.

Any other method for suppressing, decreasing or inducing endogenous gene expression known to the skilled man is also comprised within the content of this application.

Summary of the invention

The present invention relates to an isolated polynucleic acid encoding a protein or polypeptide having the biological activity of a germacrene A synthase. The invention further relates to an isolated DNA sequence encoding a protein or polypeptide with germacrene A synthase activity, having at least 70%, preferably at least 75% or 80%, more preferably at least 85% or 90%, most preferably at least 95%, especially preferably 100% sequence similarity with part or all of the amino acid sequence of SEQ ID NO 7 and/or SEQ ID NO 8, most preferably with the region of AA 271 to 455 of SEQ ID NO 7 or the region of AA 293 to 477 of SEQ ID NO 8. The invention further relates to an isolated polynucleic acid encoding a protein or polypeptide with germacrene A synthase activity, whereby the DNA sequence has at least 70%, preferably at least 75% or 80%, more preferably at least 85% or 90%, most preferably at least 95%, especially preferably 100% sequence similarity with all or part of the DNA sequence of SEQ ID NO 3 or SEQ ID NO 4.

The term "polynucleic acid" refers to DNA or RNA, or amplified versions thereof, or the complement thereof.

The invention further relates to a polynucleic acid encoding a protein or polypeptide having the biological activity of a germacrene A synthase comprising

(a) a sequence represented in SEQ ID NO 3 or 4, or,

(b) a sequence hybridizing with a sequence as defined in (a) or,

(c) a sequence which is redundant as a result of the degeneracy of the genetic code to a sequence under (a) or (b), or

(d) a complement of any of the sequences under (a), (b) or (c).

The term "hybridizing" refers to hybridization conditions as described in Sambrook (Molecular cloning, a laboratory manual, Cold Spring Harbor Press, 1989, page 7.52), preferably specific or stringent hybridization conditions are used.

The invention further relates to a method of producing germacren A or sesquiterpene lactones, which method comprises expressing a polynucleic

acid having at least 70%, preferably at least 75% or 80%, more preferably at least 85% or 90%, most preferably at least 95% similarity with, especially 100% sequence similarity to the nucleotide sequence of SEQ ID NO 3 and/or SEQ ID NO 4, encoding a polypeptide having the biological activity of a germacrene A synthase, in a suitable host cell, in the presence of farnesyl diphosphate, and, optionally, isolating the germacrene A or sesquiterpene lactones thus formed.

The invention further pertains to a recombinant polynucleic acid comprising one or more DNA sequences having at least 70% or 75%, preferably at least 80% or 85%, more preferably at least 90%, most preferably at least 95%, especially preferably 100% sequence similarity to the nucleotide sequence of SEQ ID NO 3 or SEQ ID NO 4, or the complementary strand thereof, under the control of a plant expressible promoter.
Furthermore, the invention pertains to a recombinant polynucleic acid, which is a sesquiterpenoid modulating gene (SMG), comprising one or more polynucleic acid sequences, each under control of a plant-expressible promoter, such as, but not limited to those sequences described above or parts thereof, which when expressed in a cell of a plant either induce, increase or decrease the activity of a sesquiterpenoid synthase, such as germacrene A synthase, in that cell.

The present invention also relates to probes and primers derived from the new germacrene A synthase genes that are useful for instance for the isolation of additional germacrene A synthase genes having sequences which differ from SEQ ID NO 1 to 4 by techniques known in the art, such as PCR cloning.
The term "probe" according to the present invention refers to a single-stranded oligonucleotide which is designed to specifically hybridize to any of the germacrene A synthase polynucleic acids of the invention.
The term "primer" refers to a single stranded oligonucleotide sequence capable of acting as a point of initiation for synthesis of a primer extension product which is complementary to the germacrane synthase A gene nucleic acid strand to be copied. Preferably the primer is about 5-50 nucleotides long. The term "target region" of a probe or a primer according to the present invention is a sequence within the germacrene A synthase polynucleic

acid(s) to which the probe or the primer is completely complementary or partially complementary (i.e. with some degree of mismatch). It is to be understood that the complement of said target sequence is also a suitable target sequence in some cases.

"Specific hybridization" of a probe to a target region of the germacrene A synthase polynucleic acid(s) means that the probe forms a duplex with part of this region or with the entire region under the experimental conditions used, and that under those conditions this probe does substantially not form a duplex with other regions of the polynucleic acids present in the sample to be analysed.

"Specific hybridization" of a primer to a target region of the germacrene A synthase polynucleic acid(s) means that, during the amplification step, said primer forms a duplex with part of this region or with the entire region under the experimental conditions used, and that under those conditions the primer does not form a duplex with other regions of the polynucleic acids present in the sample to be analysed. It is to be understood that "duplex" as used hereby, means a duplex that will lead to specific amplification.

Preferably, the probes of the invention are about 5 to 50 nucleotides long, more preferably from about 10 to 25 nucleotides. Particularly preferred lengths of probes include 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24 or 25 nucleotides. The nucleotides as used in the present invention may be ribonucleotides, deoxyribonucleotides and modified nucleotides such as inosine or nucleotides containing modified groups which do not essentially alter their hybridization characteristics.

Probe and primer sequences are represented throughout the specification as single stranded DNA oligonucleotides from the 5' to the 3' end. It is obvious to the man skilled in the art that any of the below-specified probes can be used as such, or in their complementary form, or in their RNA form (wherein T is replaced by U).

For designing probes with desired characteristics, the following useful guidelines known to the person skilled in the art can be applied.

The extent and specificity of hybridization reactions such as those described herein are affected by a number of factors, such as degree of complementarity, stability of the probe:target nucleic acid and hybridization conditions including ionic strength, incubation temperature and presence of chemical reagents. Manipulation of one or more of those factors will

determine the exact sensitivity and specificity of a particular probe. The importance and effect of various assay conditions are well known to the person skilled in the art.

Standard hybridization and wash conditions are disclosed in the Examples section. Other conditions are for instance 3X SSC (Sodium Saline Citrate), 20% deionized FA (Formamide) at 50□C. Other solutions (SSPE (Sodium saline phosphate EDTA), TMAC (Tetramethyl ammonium Chloride), etc.) and temperatures can also be used provided that the specificity and sensitivity of the probes is maintained. When needed, slight modifications of the probes in length or in sequence have to be carried out to maintain the specificity and sensitivity required under the given circumstances.

The invention further encompasses transgenic plants, plant organs, plant tissues or cells, obtained by introducing into their genome a recombinant polynucleic acid comprising one or more DNA sequences having at least 70% or 75%, preferably at least 80% or 85%, more preferably at least 90%, most preferably at least 95%, especially preferably 100% sequence similarity to the nucleotide sequence of SEQ ID NO 3 or SEQ ID NO 4, or the complementary strand thereof, each under the control of a plant expressible promoter.

Furthermore, the invention pertains to a recombinant polynucleic acid, which is a sesquiterpenoid modulating gene (SMG), comprising one or more polynucleic acid sequences, each under control of a plant-expressible promoter, such as, but not limited to those sequences described above or parts thereof, which when expressed in a cell of a plant either induce, increase or decrease the activity of a sesquiterpenoid synthase, such as germacrene A synthase in that cell.

The invention further encompasses transgenic plants, plant organs, plant tissues or cells, obtained by introducing into their genome a recombinant polynucleic acid comprising one or more DNA sequences having at least 70% or 75%, preferably at least 80% or 85%, more preferably at least 90%, most preferably at least 95%, especially preferably 100% sequence similarity to the nucleotide sequence of SEQ ID NO 3 or SEQ ID NO 4, or the complementary strand thereof, each under control of a plant expressible-promoter.

The invention further encompasses transgenic plants, plant organs, plant tissues or cells, having modified sesquiterpenoid synthase activity, due to the presence in their genome of one or more transgenes which, when expressed inhibit the activity of a sesquiterpenoid synthase, such as a germacrene A synthase.

The invention further encompasses transgenic plants, plant organs, plant tissues or cells, having modified taste or pathogen resistance due to the presence in their genome of one or more transgenes which, when expressed inhibit the activity of a sesquiterpenoid synthase, such as a germacrene A synthase.

The invention further encompasses transgenic plants, plant organs, plant tissues or cells, having modified taste or pathogen resistance due to the presence in their genome of one or more transgenes which, when expressed induce or increase the activity of a sesquiterpenoid synthase, such as a germacrene A synthase.

The invention further encompasses transgenic plants, plant organs, plant tissues or cells, having modified sesquiterpene lactone production due to the presence in their genome of one or more transgenes which, when expressed induce or increase the activity of a sesquiterpenoid synthase, such as a germacrene A synthase.

The invention further encompasses a process for modifying taste and/or resistance in a plant, plant organ, tissue or cell comprising introducing one or more recombinant polynucleic acids which induce, increase, decrease or inhibit the expression or activity of a sesquiterpenoid synthase, such as germacrene A synthase.

More particularly, the invention relates to a process for decreasing the bitter taste in a plant, plant organ, tissue or cell, comprising introducing into plant cells or tissues on or more recombinant polynucleic acids comprising a polynucleic acid sequence having at least 70% or 75%, preferably at least 80% or 85%, more preferably at least 90%, most preferably at least 95%,

especially preferably 100% similarity to the nucleotide sequence of SEQ ID NO 3 or SEQ ID NO 4 or parts thereof, or the complementary strand thereof, under the control of a plant-expressible promot r, regen rating the transformed plant cells or tissues into plants and obtaining the plants, plant organs, tissues or cells having decreased bitter taste.

Brief description of the drawings

The following detailed description, given by way of example, but not intended to limit the invention to specific embodiments described, may be understood in conjunction with the accompanying figures, incorporated herein by reference, in which:

Figure 1. Enzyme activity of 0.5 mL fractions eluting from a Mono-Q anion exchange column (FPLC).

Figure 2. Radio-GLC traces showing radio-labeled products of enzyme assays on A, fraction 20 (elution volume 10 mL) and B, fraction 26 (elution volume 13 mL) of the Mono-Q eluent shown in Fig. 1. The major peak in both traces represents germacrene A, the minor peaks are rearrangement products of germacrene A.

Figure 3. Radio-GLC analysis of radiolabeled products formed from [^{3}H]-farnesyl diphosphate in assays with protein extracts from transformed *E. coli* BL 21 (DE3) cells (Stratagene). A, FID signal showing an unlabelled authentic standard of germacrene A. B, C, radio-traces showing enzymatic products of protein extracts from BL 21 (DE3) cells transformed with A, the short cDNA and B, the long cDNA. **1**, selina-4, 11-diene **2,3,** α- and ß-selinene **4,** germacrene A. Long and short cDNA refer to the genes encoding the iso-enzymes of germacrene A synthase, of which the cDNA sequences are provided herein.

Figure 4. GC-MS analysis on an HP5-MS column of products formed from farnesyl diphosphate in assays with protein extracts from transformed *E. coli* BL 21 (DE3) cells (Stratagene). A, chromatogram of the negative control (v ctor without insert); B, chromatograms of B, the short cDNA; C

chromatogram of C, the long cDNA; D, an authentic standard of germacrene A. **1,** selina-4,11-diene **2,** ß-selinene **3,** α-selinene **4,** germacrene A. "Long" and "short" cDNA refer to the genes encoding the iso-enzymes of germacrene A synthase, of which the cDNA sequences are provided herein.

Figure 5. Mass spectra of main product peaks **4** from chromatograms in Figure 4; A: the "short" cDNA; B: the "long" cDNA; C: authentic standard of germacrene A.

Figure 6. GC-MS analysis on an enantioselective column (Selected Ion Monitoring-mode) of products formed from farnesyl diphosphate in assays with protein extracts from transformed *E. coli* BL 21 (DE3) cells (Stratagene) with co-injection of an authentic standard of (+)- and (-)-ß-elemene. (A,B) chromatograms of the short cDNA, with A, an injection port temperature of 150°C and B, an injection port temperature of 250°C. C,D, chromatograms of the long cDNA, with C, an injection port temperature of 150°C and D, an injection port temperature of 250°C. **1,** (+)-ß-elemene; **2,** (-)-ß-elemene; **3,** α-selinene; **4,** ß-selinene; **5,** selina-4,11-diene; **6,** germacrene A. "Long" and "short" cDNA refer to the genes encoding the iso-enzymes of germacrene A synthase, of which the cDNA sequences are provided herein.

Figure 7. Radio-GC analysis of radiolabeled products of incubations of crude extracts of etiolated seedlings of *Cichorium intybus* (A), inuline chicory (B), *Lactuca sativa* (C), radicchio (Chioggia type) (D), radicchio (Treviso type) (E), and endive (F) and *Lactuca sativa* (C) with 3H-labeled farnesyl diphosphate as substrate. Peaks: 1, germacrene A; 2, α/ß-selinene; 3, farnesol.

Figure 8. GC-MS spectrum of A, the major sesquiterpene product of an incubation of a crude extract of etiolated seedlings of *Lactuca sativa* with farnesyl diphosphate, and B, of an authentic standard of germacrene A.

Figure 9. Constructs for use in the production of transgenic plants with decreased germacrene A synthase activity. A= gene 1, B=gene 2 (or fragments thereof), A5'= 5'end of gene 1, B5' = 5' end of gene 2, Prom = promoter, T = terminator. Gene 1 and gene 2 refer to the cDNAs encoding the isoenzymes of g rmacrene A synthase, of which the sequences are

provided herein (or fragments thereof) The arrows in the boxes represent the sense and anti-sense orientation of the DNA sequence. The 5' ends comprise several hundred basepairs including part of the UTR. The promoter is preferably an enhanced 35S promoter, the terminator a nos terminator.

Figure 10. Sequence alignment of the two cDNAs (A = "short", SEQ ID NO 3; B = "long", SEQ ID NO 4) encoding germacrene A synthase isolated from chicory. Sequence alignment was done with the ClustalW program.

Figure 11. Sequence alignment of the deduced amino-acid sequences (A= "short", SEQ ID NO 7; B = "long", SEQ ID NO 8) of two iso-enzymes of germacrene A synthase in chicory. Sequence alignment was done with the ClustalW program.

Description of the invention

The term "gene" as used herein refers to any DNA sequence comprising several operably linked DNA fragments such as a promoter and a 5' untranslated region (the 5'UTR), which together form the promoter region, a coding region (which may or may not code for a protein), and an untranslated 3' region (3'UTR) comprising a polyadenylation site. Typically in plant cells, the 5'UTR, the coding region and the 3'UTR (together referred to as the transcribed DNA region) are transcribed into an RNA which, in the case of a protein encoding gene, is translated into the protein. A gene may include additional DNA fragments such as, for example, introns. As used herein, a genetic locus is the position of a given gene in the genome of a plant.

The term "polynucleic acid" refers to DNA or RNA, or amplified versions thereof, or the complement thereof.

The term "chimeric" when referring to a gene or DNA sequence is used to refer to the fact that the gene or DNA sequence comprises at least two functionally relevant DNA fragments (such as promoter, 5'UTR, coding region, 3'UTR, intron) that are not naturally associated with each other and originate, for example, from different sources. "Foreign" referring to a gene or a DNA sequence with respect to a plant species is used to indicate that the gene or DNA sequence is not naturally found in that plant species. An

endogenous plant gene is a gene that is naturally found in the concerned plant species.

As used herein the term "transgene" refers to a recombinant DNA or polynucleic acid molecule that is introduced into the genome of a plant. The term "recombinant DNA or polynucleic acid molecule" is used to exemplify and thus can include an isolated nucleic acid molecule which can be DNA and which can be obtained through recombinant or other procedures. This recombinant DNA molecule usually comprises at least one copy of at least one "gene of interest" (e.g. a recombinant DNA) which is capable of conferring one or more specific characteristics to the transformed plant. A "transgenic plant" refers to a plant comprising a transgene in the genome of all of its cells.

Expression of the transgene is used to indicate that the gene(s) of interest comprised in the transgene is expressed so as to confer on the plant one or more phenotypic traits (e.g. induced, increased or decreased sesquiterpenoid level) that were intended to be conferred by the introduction of the recombinant DNA molecule – the transforming DNA - used during transformation.

The term "sequence identity" with respect to a nucleotide sequence or an amino acid sequence, refers to the number of positions with identical nucleotides divided by the number of nucleotides in the shorter of the two sequences wherein alignment of the two sequences can be determined in accordance with the Wilbur and Lipmann algorithm (Wilbur and Lipmann, *PNAS USA*, **80**:726, 1983) using a window size of 20 nucleotides, a word length of 4 nucleotides, and a gap penalty of 4, and computer-assisted analysis and interpretation of the sequence data can be conveniently performed using programs of the intelligeneticsTM Suite (Intelligenetics Inc. CA). Sequences which are essentially identical have a sequence identity of at least about 70% or 75%, advantageously at least about 80%, such as at least about 85%, preferably at least about 90%, especially about 95%, such as at least 97%, and especially about 100%. It is clear that when RNA sequences are said to be essentially identical or identical, or have a degree of sequence identity with DNA sequences, thymidine (T) in the DNA sequence is considered to be equal to uracil (U) in the RNA sequence. Additionally or alternatively, the term "sequence similarity" with respect to a

nucleotide or amino acid sequence is intended to indicate a quantitative measure of similarity between two sequences. Sequence similarity as used herein can be measured using the alignment algorithm of the ClustalW program (Thompson et al., *Nucleic Acids Research* **22(22)**: 4673-7680, 1994). Sequences which are essentially similar have a sequence similarity of at least about 70%, advantageously at least about 75% or 80%, such as at least about 85%, preferably at least about 90%, especially about 95%, such as at least 97%, and especially about 100%.

More particularly, the sesquiterpenoid modulating genes as used herein will comprise a DNA sequence which is essentially similar, or, preferentially, essentially identical or identical to one or both of the nucleotide or amino acid sequences corresponding to germacrene A synthase disclosed herein, more specifically in the nucleotide sequence encoding, or the amino-acid sequence corresponding to the "active domain" of the enzyme. The active domain of sesquiterpenoid synthases, such as germacrene A synthase, was determined by Back and Chappell (1996, above) to stretch from about 40 amino acids before to about 140 amino acids behind the conserved DDXXD region.

Reduced or decreased bitterness as used herein refers to a decrease in bitter constituents, i.e., molecules that confer a bitter taste. In the context of the present invention, bitter constituents are sesquiterpenoid molecules that confer a bitter taste on plants, e.g., sesquiterpenoid lactones. These can be determined qualitatively and quantitatively using HPLC (Price et al., 1990, above; Van Beek et al., 1990, above). Alternatively, the activity of enzymes catalyzing the formation of intermediates in the synthesis of the bitter constituents can be used as a measure for bitterness, such as the activity of germacrene A synthase, which catalyses the production of germacrene A, an intermediate in the production of sesquiterpenoid lactones. An "increase" or "decrease" of bitterness or bitter constituents in a transgenic plant or plant part, as described herein, is determined relative to a non-transgenic plant or plant part.

Resistance as referred to herein relates to a decreased infection state of a plant by certain insects, nematodes, microorganisms or decreased feeding of vertebrat herbivores. In the context of the present invention, resistance will

primarily be the result of an increased deterrence to certain organisms, but can also be the result of an increased toxicity of the plant or plant parts to certain organisms. Alternatively, resistance of a plant or plant part can be measured by the presence therein of sesquiterpenoid molecules with deterring activity. An "increase" in resistance of a transgenic plant or plant part, as described herein, is determined relative to the resistance of a comparable non-transgenic plant or plant part.

As used herein, "modulation of sesquiterpenoids" refers to the influencing of the level of one or more sesquiterpenoids in a plant, and can thus refer either to an induction, increase or decrease of production of sesquiterpenoids in the plant. This modulation is preferably achieved, according to the invention, by influencing the level and/or activity of one or more sesquiterpenoid synthases in a plant. Sesquiterpenoid synthases are enzymes that are involved in the biosynthesis of sesquiterpenoids. A "germacrene A synthase" as used herein refers to an enzyme capable of producing germacrene A, preferably as a stable compound which is released from the enzyme without further processing by that enzyme.

The genes which according to this invention, can be used to modulate the level and/or activity of sesquiterpenoid synthases in plants will generally be referred to as "sesquiterpenoid modulating genes" ("SMGs"). These are foreign or endogenous genes encoding sesquiterpenoid synthases or transgenes derived from genes encoding sesquiterpenoid synthases, more particularly genes encoding germacrene A synthase. Modulation of sesquiterpenoid synthase activity is obtained, according to one embodiment of the invention, by influencing endogenous gene expression in the plant. This is preferably achieved by introducing into the genome of the plant, one or more transgenes which interact with the expression of endogenous genes, by anti-sense RNA, co-suppression or ribozyme suppression.

Alternatively, introduction of one or more DNA sequences encoding a sesquiterpenoid synthase into the plant genome, in a suitable conformation for gene expression (e.g. under control of a plant-expressible promoter), will result in increased or induced expression of the sesquiterpenoid synthase(s) in the plant, and, in the presence of an adequate substrate, in an increase of the corresponding s squiterpenoid.

Induced, increased or reduced expression of a sesquiterpenoid synthase gene in a transgenic plant or plant cell as compared to a non-transgenic plant or plant cell can be measured by measuring mRNA levels, or where appropriate, the level or activity of the sesquiterpenoid synthase (e.g. ELISA, activity of the enzyme as indicated by the level of sesquiterpenoid or metabolites thereof (such as sesquiterpenoid lactones) formed. Endogenous sesquiterpenoid synthase expression refers to the expression of a protein with sesquiterpenoid synthase activity which is naturally found in the concerned plant, plant part or plant cell.

The biological activity of a sesquiterpenoid synthase can be measured directly in vitro by incubation of a purified or non-purified sample with the substrate of the sesquiterpenoid synthase, preferably labeled, after which the catalytic activity of the sesquiterpenoid synthase can be measured. For example, germacrene A synthase activity in a sample can be measured by incubating a sample allegedly containing the enzyme with (radiolabeled) farnesyl diphosphate as substrate, after which production of germacrene A can be measured by radio-GC analysis or GC-MS analysis (for example as described in the examples herein).

As used herein, the term a "plant-expressible promoter" refers to a promot r that is capable of driving transcription in a plant cell. This includes any promoter of plant origin, including the natural promoter of the transcribed DNA sequence, but also any promoter of non-plant origin which is capable of directing transcription in a plant cell, i.e. certain promoters of viral or bacterial origin such as the Cauliflower Mosaic Virus 35S (CaMV35S) or the T-DNA gene promoters. The term "plant-expressible promoter" includes, but is not restricted to, constitutive, inducible, and tissue-specific promoters.

The present invention is based on the observation that the bitter taste of certain vegetables, for example chicory, is related to the synthesis of sesquiterpene lactones, for example guianolides. As decreasing the bitterness would increase the market value of some of these vegetables, the biosynthesis of the sesquiterpene lactones in chicory was investigated. It was found that germacrene A is the product of a germacrene A synthase from which it is released without being further convert d into other

sesquiterpenoids by this enzyme. Other enzymes further modify the germacrene A skeleton to produce the variety of sesquiterpene lactones present in chicory. Germacrene A synthase was partially purified and the corresponding genes were isolated. The present invention is further based on the observation that certain sesquiterpene lactones play a role in the resistance of plants against several organisms. Based on these observations the concept was developed that the genes encoding sesquiterpenoid synthases could be used to influence both flavor and resistance in plants.

As the isolated genes encoding monoterpene-, sesquiterpene- and diterpene cyclases display a strong similarity in gene-organization and in amino acid sequence within domains, it is expected that modulation of gene expression, e.g., by anti-sensing or co-suppression, may influence the expression of different genes displaying such similar structure. It should be understood that such similarities can and should be taken into account when designing the transgenes used in the present invention.

In one embodiment of the invention, the production of the bitter constituents in plants is reduced or suppressed, by modulating the level and/or activity of sesquiterpenoid synthase(s) in the plant involved in the production of guianolides and other sesquiterpenoids associated with the bitter taste in vegetables. More particularly, modulation is achieved by suppressing endogenous germacrene A synthase levels by anti-sense RNA, co-suppression or other methods of gene suppression.
In different vegetables, such as chicory, endives, radicchio, lettuce and artichoke, sesquiterpenoid lactones have been shown to be important bitter constituents. The isolation from chicory of the sesquiterpenoid synthase involved in the biosynthesis of these bitter constituents as well as the genes encoding this enzyme is described herein (example 1 to 5).

According to one aspect of the invention, a decrease in activity of a sesquiterpenoid synthase in a plant or parts of a plant is obtained by introducing into the cells of the plant one or more transgenes which are sesquiterpenoid modulating genes (SMGs), capable of influencing the level of one or more endogenous sesquiterpenoid synthase(s) in the plant. The sesquiterp noid modulating genes of the present invention comprise a

transcribed DNA sequence under the control of, and fused at its 5' end to, the plant-expressible promoter, whereby the resulting RNA, protein or polypeptide, when expressed in cells of the plant, significantly disturb or reduce the level and/or the activity of the endogenous sesquiterpenoid synthase(s). Alternatively, the stable integration of the transgene(s) into the cell in itself results in a decreased expression of the endogenous sesquiterpenoid synthase gene(s).

Thus, in one embodiment of the invention, the sesquiterpenoid modulating gene (SMG) comprises a DNA which encodes an anti-sense RNA which is complimentary to at least part of the sense mRNA of a sesquiterpenoid synthase gene that is naturally transcribed in the cells. More particularly, the SMG comprises a DNA encoding an anti-sense RNA which is the complement of the sense RNA of a germacrene A synthase gene from chicory, most particularly the complement of SEQ ID NO 3 or 4 or a part thereof. Alternatively, the SMG comprises a DNA encoding an anti-sense RNA which has at least 70% or 75%, preferably at least 80% or 85%, particularly at least 90%, more particularly 95%, especially has 100% sequence similarity to the complement of the sense RNA of the sequence of SEQ ID NO 3 or SEQ ID NO 4, or a part thereof. The anti-sense RNA may be complementary to any part of the sense mRNA (corresponding to part or all of an intron, exon, leader sequence etc., coding or non-coding region). Preferably, the anti-sense RNA is complementary to the sense RNA sequence encoding the active domain of the enzyme. More particularly, the anti-sense RNA comprises a sequence of at least 20 nucleotides, preferably 100 nucleotides complementary to the sense RNA encoded by nucleotide 845 to 1390 of SEQ ID NO 3 or to the sense RNA encoded by nucleotide 906 to 1460 of SEQ ID NO 4. Preferably, the SMG does not encode a functional protein, more particularly it does not encode a protein.

Suppression of germacrene A synthase activity may be obtained using one or more SMGs, which can comprise a DNA sequence which encodes an anti-sense RNA which is identical or similar to the complement of the sequence of SEQ ID NO 3 or part thereof, or a DNA sequence which encodes an anti-sense RNA which is identical or similar to the complement of the sequence of SEQ ID NO 4 or part thereof. Alternatively, an SMG may be used which encodes an anti-sense RNA which is at least 60%, preferably at least 65%,

most preferably at least 70% similar to the complement of a sequence between nucleotide 845 and nucleotide 1390 of SEQ ID NO 3 and of a sequence between nucleotide 906 and nucleotide 1460 of SEQ ID NO 4.

In another embodiment of the invention, the transcribed DNA sequence of the SMG comprises a DNA that is strongly homologous or similar to an endogenous sesquiterpenoid synthase sequence, so that introduction of the SMG into the genome of the plant causes the endogenous sesquiterpenoid synthase gene to be suppressed (co-suppression). Suppression of expression of the endogenous gene is achieved by introduction of a SMG comprising a strong plant-expressible promoter operably linked to a DNA so that the resulting transcribed RNA is a sense RNA comprising a nucleotide sequence which has at least 75%, preferably at least 80%, particularly at least 85%, more particularly at least 90%, especially at least 95% similarity with, more especially has 100% sequence similarity to the coding or transcribed DNA sequence (sense) of the endogenous sesquiterpenoid synthase gene of which the expression is to be suppressed. Particularly, The SMG comprises a DNA that displays a sequence similarity with the transcribed DNA region of a germacrene A synthase gene or a part thereof. More particularly, it comprises a DNA with a sequence similarity to the transcribed DNA region of SEQ ID NO 3 or SEQ ID NO 4. Particularly, the transcribed region of the SMG does not code for a protein. Preferably, the transcribed DNA region of the SMG does not code for a functional protein. Suppression of germacrene A synthase activity may be obtained using one or more SMGs, which can comprise one or more DNA sequences which is identical or essentially similar the sequence of SEQ ID NO 3 or part thereof, or identical or essentially similar to the sequence of SEQ ID NO 4 or part thereof. Alternatively, an SMG may be used which has at least 70% or 75%, preferably at least 80%, most preferably at least 90%, especially at least 95% similarity with, more especially has 100% sequence similarity to a sequence between nucleotide 845 and nucleotide 1390 of SEQ ID NO 3 and to a sequence between nucleotide 906 and nucleotide 1460 of SEQ ID NO 4.

Recently, Waterhouse et al. (*PNAS*, **95(23)**: 13959-64, 1998) have described methods and means to make gene silencing in plants more efficient and predictable, by simultaneous expression of both sense and anti-sense constructs in cells of one plant. The sense and anti-sense nucleic acids may

be in the same transcriptional unit, so that a single RNA transcript that has self-complementarity is generated upon transcription.
In an analogous way, Hamilton et al. (*The Plant Journal* 15(6): 737-746, 1998) describe improved silencing e.g. of tomato ACC-oxidase gene expression using a sense RNA containing two additional upstream inverted copies of its 5' untranslated region.
WO 98/53083 describes constructs and methods for enhancing the inhibition of a target gene within an organism, involving the insertion into the gene silencing vector of an inverted repeat of all or part of a polynucleotide region within the vector.

In a further embodiment of the invention, an SMG comprises a DNA, which encodes a specific RNA enzyme or ribozyme, capable of highly specific cleavage of an endogenous sesquiterpenoid synthase gene of a plant. Particularly, the ribozyme encoded by the DNA is targeted against a gene encoding germacrene A synthase, most particularly against the mRNA sequence corresponding to the cDNA of SEQ ID NO 3 and/or SEQ ID NO 4.

It is understood that alternative methods can be developed for decreasing the sesquiterpenoid synthase activity in plants or plant parts in order to reduce bitterness, for instance inhibition the activity of the enzyme itself. Thus, the present invention also relates to sesquiterpenoid modulating genes encoding a protein or polypeptide capable of inhibiting the activity of a sesquiterpenoid synthase, more particularly, capable of inhibiting germacrene A synthase activity. Such an SMG can encode, for instance, an antibody or a fragment of an antibody directed against a germacrene A synthase. More particularly, the antibody fragment will be directed against an epitope of the protein made up of the sequence of SEQ ID NO 7 and/or SEQ ID NO 8,

According to another aspect of the invention, sesquiterpenoid modulating genes (SMG), are used to increase or induce expression of sesquiterpenoid synthase in a plant, so as to increase the level of sesquiterpenoids conferring resistance to the plant or increase the level of sesquiterpenoids that are interesting for other reasons, for example flavor and fragrance compounds derived from germacrene A or sesquiterpene lactones with pharmacological activity. This is achieved by introducing into the genome of a plant one or

more SMGs comprising a DNA encoding a protein with sesquiterpenoid synthase activity, under the control of a plant-expressible promoter. More specifically, the SMG comprises a DNA encoding protein with germacrene A synthase activity. For instance, the SMG can comprise a sequence encoding a protein which has at least 70% or 75%, preferably at least 80% or 85%, most preferably 90%, especially at least 95% similarity with, more especially has 100% sequence similarity to SEQ ID NO 7 and/or SEQ ID NO 8, or a part thereof, encoding a functional part of a germacrene A synthase. Plants particularly suited for this embodiment of the invention are plants already producing sesquiterpene lactones such as for example many of the members of the Asteracea, such as species from the genera *Cichorium, Lactuca,* and *Helenium* (in case upregulation of sesquiterpene lactone formation is required), or plants already producing sesquiterpenes such as for example the genera *Carum, Capsicum, Chamomilla, Cichorium, Citrus, Daucus, Gossypium, Juniperus, Lactuca, Tanacetum, Lycopersicon, Nicotiana, Pogostemon, Vetiveria* (in case the production of germacrene A or other terpenoids derived thereof such as nootkatone is desired). If a high production is required, or when a shortage in FDP, the enzyme's substrate, is anticipated, a recombinant DNA encoding a protein or polypeptide with germacrene A synthase activity may be combined with a DNA encoding a protein with FDP synthase activity.

According to the invention, sesquiterpenoid synthase expression and/or activity in a plant or in plant parts is modulated by introducing one or more sesquiterpenoid modulating genes (SMGs) into the genome of the plant. The SMG(s) comprise(s) a coding region placed under the control of, and fused at its 5' end to, a plant-expressible promoter. This promoter can be the natural promoter of an endogenous sesquiterpenoid synthase gene, more particularly the promoter of an endogenous germacrene A synthase gene, most particularly the promoter of a gene corresponding to the cDNA of SEQ ID NO 3 or SEQ ID NO 4.

Alternatively, the SMG is placed under control of a constitutive promoter, directing expression in essentially all cells of the plant. More specifically, the constitutive promoter can be, but is not restricted to, one of the following: a 35S promoter (Od II et al., *Nature* **313**:482-493, 1985), a 35S'3 promoter (Hull and Howell, *Virology* **86**:482-493, 1987), the promoter of the nopaline

synthase gene ("PNOS") of the Ti-plasmid (Herrera -Estrella, *Nature* **303**:209-213, 1983) or the promoter of the octopine synthase gene ("POCS", De Greve et al., *J. Mol. Appl. Genet.* **1**(6): 499-511, 1982). It is clear that other constitutive promoters can be used to obtain similar effects.

For specific embodiments of this invention, the use of inducible promoters can provide certain advantages. In one embodiment of the invention, modulation of sesquiterpenoid synthase activity is required at least in, but possibly only in certain parts of the plant, making it possible to limit modulation to a certain period of culture or developmental stage of the plant. More particularly, it may be desired to decrease the sesquiterpenoid synthase(s) in the plant specifically in those parts of the plant destined for consumption or processing. More specifically, in a preferred embodiment of the invention the bitterness of chicory is decreased, at least in the shoots (eaten as vegetables) and/or roots (used as a source for sugars and/or feed for cattle). The shoots and roots of chicory are grown under dark conditions. Thus, in one aspect of the invention, the sesquiterpenoid modulating gene(s), is (are) placed under the control of a promoter which directs expression in the cells of the plant under specific dark conditions.
Alternatively, for other bitter tasting plants of which the leaves are used as food crop, such as, but not limited to, the lettuce (*Lactuca sativa*), light-inducible promoters can be used. Examples of inducible promoters are the dark regulated PRB-1b protein promoter described by Sessa et al. (*Plant Mol. Biol.*, **28**(3): 537-547, 1995) and the dark and light regulated chlorophyll A/B binding protein promoters, described by Cashmore (*Proc. Natl. Acad. Sci.* **81**:2960-2964, 1984) and by Simpson, et al. (*EMBO J.* **4**:2723-2729, 1985) and in US patent n° 5,656,496.
Similarly, an inducible increase in sesquiterpenoid production can be of interest to protect plants from insects, fungi, nematodes or vertebrate herbivores by placing the SMG(s) under the control of an insect- fungus-, nematode-, or wounding-inducible promoter.

For specific embodiments of this invention, the use of tissue-specific promoters can provide certain advantages. More particularly, reduction of bitterness in plants will mainly be of value for parts of the plants destined for consumption or processing. Thus, in specific embodiments of the invention,

the SMG(s) is (are) placed under the control of a promoter directing expression in specific plant tissues, such as roots or leaves. For instance, in chicory, reduction of the activity of sesquiterpenoid synthase, more particularly germacrene A synthase, is directed at the shoots (eaten as vegetables) and/or the roots (used as a source for sugars).
Similarly, in plants where certain parts of the plants are particularly susceptible to the damage of insects, microorganisms, nematodes or vertebrate herbivores, tissue-specific increase in sesquiterpenoid production can be of interest. For instance, to protect plants from infection by aphids, an increase in sesquiterpenoid synthase production is directed to the phloem or the chlorophyll-producing plant parts. More particularly, the SMG(s) is (are) placed under the control of a phloem-specific promoter (such as the rolC promoter of Agrobacterium) or the promoter of the gene encoding the small subunit of Rubisco. Alternatively, to protect plants from infection by root pathogens, for example fungi or nematodes, the increase in sesquiterpenoid synthase production is directed to the roots. More particularly, the SMG(s) is(are) placed under the control of a root-specific promoter (such as described by Keller et al., *Genes Devel.* 3: 1639-1646, 1989).

The sesquiterpenoid modulating gene(s) may include further regulatory or other sequences from other plant or bacterial genes, such as leader sequences (e.g. the cab22 leader from Petunia), 3'transcription termination and polyadenylation signals (e.g. from the octopine synthase gene or the nopaline synthase gene), plant translation initiation consensus sequences, introns etc, which is or are operably linked to the SMG.

The recombinant DNA comprising one or more SMGs may be accompanied by a chimeric marker gene. The chimeric marker gene can comprise a marker DNA that is operably linked at its 5' end to a plant-expressible promoter, preferably a constitutive promoter, such as the CaMV 35S promoter, or a light inducible promoter such as the promoter of the gene encoding the small subunit of Rubisco; and operably linked at its 3' end to suitable plant transcription 3' end formation and polyadenylation signals. It is expected that the choice of the marker DNA is not critical, and any suitable marker DNA can be used. For example, a marker DNA can encode a protein that provides a distinguishable color to the transformed plant cell, such as the

A1 gene (Meyer et al., *Nature* **330**: 677, 1987), can provide herbicide resistance to the transformed plant cell, such as the *bar* gene, encoding resistance to phosphinothricin (EP 0,242,246), or can provide antibiotic resistance to the transformed cells, such as the *aac(6')* gene, encoding resistance to gentamycin (WO94/01560).

The cell of a plant is preferably transformed in accordance with this invention, using a vector that is a disarmed Ti-plasmid containing the transgene and carried by *Agrobacterium*. This transformation can be carried out using the procedures described, for example, in European patent publications 0,116,718 and 0,270,822. Protocols describing *Agrobacterium*-mediated transformation of lettuce, chicory and tobacco are described in Michelmore, R. et al. (*Plant Cell Reports* **6,** 439-442, 1987), Hohn and Ohlrogge, (*Plant Physiology* **97,** 460-462, 1991) and Frulleux et al. (*Plant Cell, Tissue and Organ Culture* **50,** 107-112, 1997). Preferred Ti-plasmid vectors contain the transgene sequence between the border sequences, or at least located to the left of the right border sequence, of the T-DNA of the Ti-plasmid. Where advantageous, plants are preferably transformed with auxotrophic *Agrobacterium* strains as described in European Patent Application 9711465.3). Of course other methods can be used to transform the plant cell, such as direct gene transfer (as described, for example in EP 0,223,247), pollen-mediated transformation (as described, for example in EP 0,270,356, WO85/01856), in vitro protoplast transformation (as described for example in US patent 4,684,611), plant RNA virus-mediated transformation (as described, for example in European patent publication 0,067,553 and US patent 4,407,956) and liposome mediated transformation (as described, for example, in US patent 4,536,475).

Although it is clear that the invention can be applied essentially to all plant species and varieties, the invention will be especially suited for those plants for which a decrease in bitter constituents or an increased resistance would result in an enhanced commercial value. The obtained transformed plants can be used in a conventional breeding scheme to produce more transformed plants with the same characteristic or to introduce the modified sesquiterpenoid synthase activity characteristic of the invention in other varieties of th same or related plant species. Seeds obtained from the

transformed plants contain the transgene of the invention as a stable genomic insert.

The following Examples describe the isolation of a novel sesquiterpenoid synthase gene, the germacrene A synthase gene from *Cichorium intybus* and the use of this sequence or parts thereof in the manipulation of germacrene A synthase activity in plants. Unless stated otherwise in the Examples, all recombinant DNA techniques are carried out according to standard protocols as described in Sambrook et al. (1989) Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, NY and in volumes 1 and 2 of Ausubel et al. (1994) Current Protocols in Molecular Biology, Current Protocols, USA. Standard materials and methods for plant molecular work are described in Plant Molecular Biology Labfax (1993) by R.D.D. Croy, jointly published by BIOS Scientific Publications Ltd. (UK) and Blackwell Scientific Publications, UK.

Throughout the Description and Examples reference is made to the following sequences:

SEQ ID NO 1: PCR fragment 1 (short)
SEQ ID NO 2: PCR fragment 2 (long)
SEQ ID NO 3: cDNA 1 encoding C. intybus germacrene A synthase (short)
SEQ ID NO 4: cDNA 2 encoding C. intybus germacrene A synthase (long)
SEQ ID NO 5: amino acid sequence encoded by PCR fragment of SEQ ID NO 1
SEQ ID NO 6: amino acid sequence encoded by PCR fragment of SEQ ID NO 2
SEQ ID NO 7: amino acid sequence encoded by cDNA of SEQ ID NO 3
SEQ ID NO 8: amino acid sequence encoded by cDNA of SEQ ID NO 4
SEQ ID NO 9: primer
SEQ ID NO 10: primer
SEQ ID NO 11: primer
SEQ ID NO 12: primer
SEQ ID NO 13: primer
SEQ ID NO 14: primer

Example 1: Partial purification from chicory of two proteins catalyzing the formation of germacrene A from FDP

Chicory chicons were cut into small pieces, frozen in liquid nitrogen and ground to a fine powder using a cooled mortar and pestle. One gram of this powder was homogenized in 10 mL buffer containing 25 mM Mopso (pH 7.0), 20% (v/v) glycerol, 25 mM sodium ascorbate, 25 mM $NaHSO_3$, 10 mM $MgCl_2$ and 5 mM DTT (buffer A), slurried with 0.5 g polyvinylpolypyrrolidone (PVPP) and a spatula tip of purified sea sand. To the homogenate 0.5 grams of polystyrene resin (Amberlite XAD-4, Serva) were added and the slurry was stirred carefully for 10 min and then filtered through cheesecloth. The filtrate was centrifuged at 20,000*g* for 20 min (pellet discarded), and then at 100,000*g* for 90 min. The 100,000*g* supernatant was loaded on a 10 x 2.5 cm column of Q-sepharose (Pharmacia Biotech) previously equilibrated with buffer containing 15 mM Mopso (pH 7.0), 10% (v/v) glycerol, 10 mM $MgCl_2$ and 2 mM DTT (buffer B). The column was washed with the equilibration buffer and eluted with a 0-2.0 M KCl gradient. For determination of enzyme activities, 20 µL of the 2.0-ml fractions were diluted 5-fold in an Eppendorf tube with buffer B and 20 µM [^{3}H]FDP was added. The reaction mixture was overlaid with 1 mL of hexane to trap volatile products and the contents mixed. After incubation for 30 min at 30°C, the vials were mixed, and centrifuged to separate phases. A portion of the hexane phase (750 µL) was transferred to a new Eppendorf tube containing 40 mg of silica gel, and, after mixing and centrifugation, 500 µL of the hexane layer was removed for liquid scintillation counting in 4.5 ml of Ultima Gold cocktail (Packard).

The combined active fractions were desalted to buffer B, and 1.0 mL of this enzyme preparation was applied to a Mono-Q FPLC column (HR5/5, Pharmacia Biotech), previously equilibrated with buffer B containing 0.1% Tween-20. The column was eluted with a gradient of 0-600 mM KCl in th same buffer. The activity, which eluted as one peak from the Q-sepharose column, was now separated into two activity peaks (activity assessed as described above) (Fig. 1). For determination of product identity, 0.5 mL of the two most active fractions were diluted 2-fold with buffer B and 20 µM [^{3}H]-FDP were added. After the addition of a 1-mL redistilled pentane overlay, the tubes were carefully mixed and incubated for 1 h at 30°C. Following the assay, the tubes were mix d, the organic layer was removed and passed over a short column of aluminum oxide overlaid with anhydrous $MgSO_4$. The

assay was extracted with another 1 mL of diethyl ether, which was also passed over the aluminum oxide column, and the column washed with 1.5 mL of diethyl ether. Before radio-GLC-analysis the extract was carefully concentrated under a stream of N_2.

Radio-GLC was performed on a Carlo-Erba 4160 Series gas chromatograph equipped with a RAGA-90 radioactivity detector (Raytest, Straubenhardt, Germany). Sample components eluting from the column were quantitatively reduced before radioactivity measurement by passage through a conversion reactor filled with platinum chips at 800°C. Samples of 1 µL were injected in the cold on-column mode. The column was a fused silica capillary (30 m x 0.32 mm i.d.) coated with a film of 0.25 µm of polyethylene glycol (EconoCap EC-WAX, Alltech Associates) and operated with a He-flow of 1.2 mL min^{-1}. The oven temperature was programmed to 70°C for 5 min, followed by a ramp of 5° min^{-1} to 210°C and a final time of 5 min. About 20% of the column effluent was split with an adjustable splitter to an FID (temperature 270°C). The remainder was directed to the conversion reactor and radio detector. H_2 was added prior to the reactor at 3 mL min^{-1}, and CH_4 as a quench gas prior to the radioactivity detector (5 mL counting tube) to give a total flow of 36 mL min^{-1}. Both fractions showed exactly the same radiolabeled products, with the major peak belonging to germacrene A (Fig. 2), proving that chicory contains two distinct proteins both catalyzing the formation of germacrene A from FDP. The minor peaks preceding the germacrene A peak, belong to rearrangement products of germacrene A, *viz.* α/ß-selinene, and selina-4,11-diene (De Kraker et al., 1998, above).

Example 2: Isolation of the genes encoding germacrene A synthase

a) Isolation of mRNA. Total RNA was isolated from chicory chicons using the purescript RNA isolation kit (Biozym). DNase I (Deoxyribonuclease I, RNase free) was used to remove DNA from the RNA isolate. The DNase I was removed with a phenol/chloroform extraction after which the RNA was precipitated (ethanol precipitation with NaAc). Poly(A) + RNA was extracted from 20 µg of total RNA using 2 µg poly-d(T)25V oligonucleotides coupled to 1 mg paramagnetic beads (Dynal A.S.). The poly(A) + RNA was resuspended in 20 µl H_2O.

b) cDNA synthesis. The reverse transcription reaction was carried out in a 50 µl reaction containing 10 µl poly (A) + mixture, 0.3 µg oligo $(dT)_{25}V$, 1 mM each dATP, dTTP, dCTP and dGTP, 50 mM Tris-HCl pH=8.3, 80 mM KCl, 10 mM MgCl2 and catalyzed with 12 U AMV reverse transcriptase (Pharmacia). After an incubation for 2h at 42°C the reaction was stopped and the cDNA purified with the Wizard PCR Preps DNA purification system (Promega). The cDNA was resuspended in 50 µl H_2O.

c) PCR-based probe generation.

Based on comparison of sequences of terpenoid synthases, two degenerated primers were designed for two conserved regions (Yuba et al., *Archives of Biochemistry and Biophysics*, **332:** 280-287,1996).

sense primer (primer A):

5'-TTY CAR GAY GAR AAY GGI AAR TTY AAR GA-3' (SEQ ID NO 9)

wherein Y=C/T and R=G/A

anti-sense primer (primer B):

5'-CC RTA IGC RTC RAA IGT RTC RTC -3' (SEQ ID NO 10)

wherein Y=C/T and R=G/A

PCR was performed in a total volume of 50 µl containing 0.5 µM of each of the two primers, 0.2 mM dNTP, 1 U Super Taq polymerase / 1x PCR buffer (HT Biotechnology LTD, Cambridge, England) and 10 µl cDNA. The reaction mixture was incubated in a thermocycler (Robocycler, Stratagene) with 1 min denaturation at 94°C, 1.5 min annealing at 42°C and 1 min elongation at

72°C during 40 cycles. Agarose gel electrophoresis revealed a single specific PCR product of approximately 550 bp. The PCR product was purified using the Wizard PCR Preps DNA purification system (Promega) and subcloned using the pGEMT system. *E. coli* JM101 was transformed with this construct. 12 individual transformants were sequenced which resulted in two different sequences (SEQ ID NO 1 and SEQ ID NO 2, the deduced amino acid sequences (primers included) are shown in SEQ ID NO 5 and 6, respectively).

d) cDNA library construction and screening.

A cDNA library was constructed using the UniZap XR custom cDNA library service (Stratagene). For library screening 200 ng of both PCR amplified probes were gel-purified, randomly labeled with [α-^{32}p]dCTP, according to manufacturer's recommendation (Ready-To-Go DNA labeling beads (-dCTP), Pharmacia) and used to screen replica filters of 10^4 plaques of the cDNA library plated on *E. coli* XL1-Blue MRF' (Stratagene). The plaque lifting and hybridization were carried out according to standard protocols. Positive clones were isolated using a second and third round of hybridization. *In vivo* excision of the pBluescript phagemid from the Uni-Zap vector was performed according to manufacturer's instructions (Stratagene). Two groups of positive clones were obtained which could be distinguished using restriction enzymes and specific PCR primers. The cDNA sequences of two representatives of the two groups are shown in SEQ ID NO 3 and 4. These were named the "short" and "long" germacrene A synthase cDNAs (also referred to as gene 1 and gene 2 or A and B). The deduced amino acid sequences are shown in SEQ ID NOs 7 and 8, respectively. Nucleotide and amino acid sequence alignments are shown in Figures 10 and 11 respectively.

Example 3: Expression of the isolated genes in *E. coli*

For functional expression, the cDNA clones were subcloned in frame into the expression vector pET 11d (Stratagene). To introduce suitable restriction sites for subcloning, gene A was amplified by PCR using the following sense and anti-sense primers:

sense primer:

5'-CAA TCC GAA CCA TGG CTC TCG TT-3' (SEQ ID NO 11)

(introducing an *Nco*I site at the start codon **ATG**)

anti-sense primer:
5'- CAC CAA ATG GAT CCA AAT TCG C-3' (SEQ ID NO 12)
(introducing a *BamH*I site behind the stop codon **TGA**).

Gene B was amplified by PCR using the following sense and anti-sense primers:

sense primer:
5'-CCT TCA AGC CAT GGC AGC AGT TG-3' (SEQ ID NO 13)
(introducing an *Nco*I site at the start codon **ATG**)

anti-sense primer:
5'-TTG TAA TAG GAT CCA CTA TAG G-3' (SEQ ID NO 14)
(introducing a *BamH*I site behind the stop codon **TGA**)

The PCR reaction was performed under standard conditions. After digestion with *BamH*I and *Nco*I, the PCR product and the expression vector pET 11d were gel purified and ligated.
The two constructs and pET 11d without an insert (as negative control) were transformed to *E. coli* BL 21 (DE3) (Stratagene), and grown overnight on LB agar plates supplemented with ampicillin at 37°C. Cultures of 50 ml LB medium supplemented with ampicillin (100 µg/ml) and 0.25 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) were occulated with these over night cultures to A_{600} = 0.5 and grown for 3 h at 27°C. The cells were harvested by centrifugation during 8 minutes at 2000 g and resuspended in 1.2 ml buffer B containing 1 mM sodium ascorbate (buffer C). The resuspended cells were sonicated on ice during 4 min (5 sec on, 30 sec off), centrifuged for 5 minutes at 4°C (14.000 rpm) and the supernatant used for assays.

Example 4: Verification of product identity of cDNAs expressed in *E. coli*

For determination of product identity, 20 µM [^{3}H]-FDP was added to 0.5 mL of the enzyme preparations diluted 1:1 with buffer C containing 0.1% tween-20. After the addition of a 1-mL redistilled pentane overlay, the tubes were

carefully mixed and incubated for 1 h at 30°C. Following the assay, the tubes were mixed, the organic layer was removed and passed over a short column of aluminum oxide overlaid with anhydrous $MgSO_4$. The assay was re-extracted with 1 mL of pentane:diethyl ether (80:20), which was also passed over the aluminum oxide column, and the column washed with 1.5 mL of pentane:diethyl ether (80:20). The extract was analyzed using radio-GLC as described above (Example 1) and using GC-MS as described below. Radio-GLC analysis showed that both cDNAs formed functionally active proteins catalyzing the formation of three or more radiolabeled sesquiterpenes from [^{3}H]-FDP (Fig. 3). The negative control (vector without insert) produced no radioactivity. The samples were also analyzed by GC-MS using a HP 5890 series II gas chromatograph equipped with an HP-INNOWax column (30 m x 0.25 mm i.d., 0.25 µm df) and HP 5972A Mass Selective Detector (Hewlett-Packard). The oven was programmed at an initial temperature of 70°C for 1 min, with a ramp of 5°C min^{-1} to 210°C and final time of 5 min. The injection port (splitless mode), interface and MS source temperatures were 150, 290 and 180°C, respectively, and the He inlet pressure was controlled by electronic pressure control to achieve a constant column flow of 1.0 mL/min. Ionization potential was set at 70 eV, and scanning was performed from 30-250 amu. The negative control produced no sesquiterpenes (Fig 4(a)), whereas in assays with the expression products of both the short (Fig. 4(b)) and the long cDNA (Fig. 4(c)) four different sesquiterpenes could be detected: selina-4,11-diene (**1**), ß-selinene (**2**), α-selinene (**3**), and germacrene A (**4**) as major product. The identity of the latter was confirmed by analysis of an authentic standard of germacrene A (courtesy of Dr W.A. König) (Fig. 4(d)), and comparison of the mass spectra with the authentic standard (Fig. 5). The other three products **1,2,3** are rearrangement products of germacrene A, i.e. they are not produced enzymatically (Teisseire, P.J., *Chemistry of fragrant substances*, VCH Publishers Inc., USA, 1994; De Kraker, J-W. et al., *Plant Physiology* **117:** 1381-1392, 1998).

The chirality of the germacrene A produced by the two genes was assessed by GC-MS using the fact that the high-temperature-induced Cope-rearrangement of germacrene A to ß-elemene occurs with retention of stereochemical configuration at C7 (De Kraker et al, 1998, above). GC-MS analysis was carried out essentially as described above, but the GC was equipped with a 25 m (0.25 mm i.d.) heptakis (6-O-TBDMS-2,3-di-O-methyl)-

β-cyclodextrin (50% in OV17) column that is able to separate the enantiomers of ß-elemene (König et al., *J. High Resolut. Chromatogr.* **17:** 315-320, 1994). The oven temperature was programmed to 45°C for 4 min followed by a ramp of 2°C min^{-1} to 170°C, and spectra were recorded in scan or Selected Ion Monitoring mode (m/z 121, 147 and 189). The injection port temperature was either 150°C (no Cope-rearrangement of germacrene A) or 250°C (Cope-rearrangement of germacrene A to ß-elemene). A standard of (+)- and (-)-ß-elemene was co-injected with the germacrene A produced by the two clones. Figs 6A and C show the chromatograms of the short and the long clone, respectively, with co-injection of (+)- and (-)-ß-elemene at an injection port temperature of 150°C. Germacrene A (**6**) is by far the major product, with small amounts of the proton-induced rearrangement products α-selinene (**3**), ß-selinene (**4**) and selina-4,11-diene (**5**). The two enantiomers of ß-elemene are separated: (+)-ß-elemene (**1**) and (-)-ß-elemene (**2**). When the injection port temperature is increased only the (-)-enantiomer of ß-elemene is formed from the germacrene A of both clones. This was demonstrated using scan measurements also without co-injection with the ß-elemene standard (data not shown). Upon co-injection with the (+)- and (-)-ß-elemene standard and using a high injection port temperature, (only) the (-)-ß-elemene peak areas increase for both clones (Figs 6B,D). This proves that the germacrene A produced by both clones is exclusively rearranged to (-)-ß-elemene, implying that both clones produce exclusively (+)-germacrene A (De Kraker et al, 1998, above). Thus it was concluded that the two sequences encode iso-enzymes of germacrene A synthase (also referred to as "long" and "short" or A and B).

Example 5: Germacrene A synthase activity in other plants

In addition to chicory, several other crops belonging to the Compositae have been shown to contain bitter sesquiterpene lactones (Price et al., 1990, above). It was therefor investigated whether germacrene A synthase activity could be detected in other lettuce, endive and chicory varieties containing these lactones. Etiolated seedlings of seven different varieties were grown for a period of 8 days at 20°C in darkness after which an enzyme extract was made of the seedlings. These extracts were incubated with radiolabeled farnesyl diphosphate, the ubiquitous precursor of sesquiterpenes. Radio-GC analysis was performed on these samples.

All seven samples displayed germacrene A synthase activity (peak 1, Figure 7). The identity of peak 1 was confirmed using GC-MS, of which one example is shown in Figure 8. The fronting of peak 1 and 2 represents selina-4,11-diene and α- and β-selinene, acid-induced rearrangement products of germacrene A (De Kraker et al., 1998, above) Peak 3 represents farnesol, produced from FDP by non-specific phosphohydrolase activity. These results strongly suggest that also in other lettuce, endive and chicory varieties, sesquiterpene lactone biosynthesis proceeds via the central intermediate germacrene A.

Example 6: Transformation of lettuce and chicory with the germacrene A synthase gene(s) to obtain reduce bitterness

Based on the results described above, the sequences encoding germacrene A synthase are used to make transgenic plants showing reduced germacrene A synthase activity.

The down-regulation of genes in plants is achieved in different ways, e.g. through sense and anti-sense inactivation. In order to inhibit the activity of endogenous germacrene A synthases to obtain plant species with decreased production of germacrene A or products derived thereof, for example sesquiterpene lactones, plant species such as chicory and lettuce (and other sesquiterpene lactone producing species) are transformed by the techniques described above using plasmids containing genes coding for the germacrene A synthase(s) in either sense (to obtain co-suppression) or anti-sense orientation.

The activity of both genes in chicory may not be inhibited using a construct bearing one of the two sequences alone. The coding regions of the two genes encoding isoenzymes of germacrene A synthase of the present invention have a sequence similarity of 67%. Within the region encoding what is believed to be the "active domain" of the enzyme (stretching from about 40 amino acids before to about 140 amino acids behind the conserved DDXXD sequence) the sequence similarity between the two genes is 82.4%. Therefore, constructs are developed which comprise more than one DNA sequence whereby one DNA sequence comprises a DNA encoding an RNA with a sequence similarity to all or part of th sequence of SEQ ID NO 3, or

the complementary strand thereof and the other encodes an RNA sequence with a sequence similarity to all or part of the sequence of SEQ ID NO 4, or the complementary strand thereof. The nucleic acid molecule used preferably comprises two sequences whereby the first sequence is identical to the complement of the second sequence, possibly separated by a spacer sequence, so as to form inverted repeats. The DNA sequences are placed under the control of adequate promoters, such as the 35S promoter, and terminator sequences and are introduced into chicory and lettuce by Agrobacterium mediated transformation.

Examples of the DNA sequences comprise:

- the DNA of SEQ ID NO 3 and 4 (or parts thereof), behind a suitable promoter and upstream of a suitable terminator
- the DNA of SEQ ID NO 3 and 4 (or parts thereof), each behind a suitable promoter and upstream of a suitable terminator
- the DNA of SEQ ID NO 3 and 4 (or parts thereof), without a promoter
- two copies of SEQ ID NO 3 and 4 (or parts thereof) behind a promoter and upstream of a terminator
- a fragment of one of the two DNA's of SEQ ID NO 3 or SEQ ID NO 4 (or parts thereof) exhibiting a sequence similarity of preferably ≥80% to the other DNA of SEQ ID NO 3 or 4, behind a promoter and upstream of a terminator
- both DNA's of SEQ ID NO 3 and SEQ ID NO 4 (or parts thereof) and an additional upstream inverted copy of the 5' end (with or without the untranslated region) of one or of both, each behind a suitable promot r (or together behind one suitable promoter, such as a 35S promoter) and upstream of a suitable terminator (Hamilton et al., 1998, above; Waterhouse et al., 1998, above). Examples of such panhandle constructs are illustrated in Figure 9 (A, B, C).
- the cDNAs of SEQ ID NO 3 and SEQ ID NO 4, or a fragment thereof, displaying at least 70% homology to each other, whereby one is place in sense and one in anti-sense orientation, placed between a suitable promoter and terminator (Figure 9(D)).

The activity of the promoter, and hence the effectiveness of the co-suppression may be increased by including suitable enhancer elements behind the promot r, upstream of the coding sequence(s).

Transgenic chicory and lettuce plants comprising the chimeric genes of the invention exhibit reduced levels of germacrene A and contain lower amounts of bitter sesquiterpene lactones.

Example 7: Transformation of lettuce and tobacco with the germacrene A synthase gene(s) to obtain (increased) germacrene A or sesquiterpene lactone formation

For the upregulation of the production of sesquiterpene lactones or for the production of germacrene A in transgenic plants, plants are transformed using plasmids comprising a sesquiterpenoid synthase modulating gene comprising a sequence encoding protein or polypeptide having germacrene A synthase activity, placed under the control of a suitable plant-expressible promoter, such as a constitutive promoter. Plasmids containing these genes can be easily transferred to lettuce and tobacco using *Agrobacterium*-mediated transformation.

Transgenic plants comprising a sesquiterpernoid synthase modulating gene encoding a protein or polypeptide with germacrene synthase A activity display an increased resistance to aphids and insects, and/or an increased production of sesquiterpene lactones.

Claims

1. An isolated polynucleic acid comprising a nucleotid sequence encoding a protein or polypeptide having the biological activity of a germacrene A synthase.

2. The isolated polynucleic acid of claim 1, wherein said nucleotide sequence encodes a protein or polypeptide with an amino acid sequence having at least 70% sequence similarity to the sequence of SEQ ID NO 7 or SEQ ID NO 8.

3. The isolated polynucleic acid of claim 2, wherein said nucleotide sequence encodes a protein or polypeptide with an amino acid sequence having at least 70% sequence similarity with the sequence of SEQ ID NO 7, between amino acid 271 and amino acid 455, or with the sequence of SEQ ID NO 8, between amino acid 291 and 477.

4. The isolated polynucleic acid of claim 1, wherein said nucleotide sequence encodes the amino acid sequence of SEQ ID NO 7.

5. The isolated polynucleic acid of claim 1, wherein said nucleotide sequence encodes the amino acid sequence of SEQ ID NO 8.

6. The isolated polynucleic acid of claim 1, wherein said nucleotide sequence has at least 70% sequence similarity to the nucleotide sequence of SEQ ID NO 3 or SEQ ID NO 4, or the complement thereof.

7. The isolated polynucleic acid of claim 1, wherein said nucleotide sequence has at least 95% sequence similarity to the nucleotide sequence of SEQ ID NO 3 or SEQ ID NO 4, or the complement thereof.

8. The isolated polynucleic acid of claim 7, wherein said nucleotide sequence is the nucleotide sequence of SEQ ID NO 3, or the complement thereof.

9. The isolated DNA of claim 7, wherein said nucleotide sequence is the nucleotide sequence of SEQ ID NO 4, or the complement thereof.

10. A process for producing a plant with modified sesquiterpenoid synthase activity, said process comprising introducing into the genome of a plant cell a recombinant DNA which when expressed in a plant cell modifies the expression of a sesquiterpenoid synthase encoded by a polynucleic acid of any one of claims 1 to 9 in said cell.

11. A process for producing a plant with reduced bitterness in some or all of its plant parts, said process comprising reducing the expression of an endogenous sesquiterpenoid synthase gene in said plant.

12. The process of claim 11, comprising

(a) introducing into the genome of a plant cell one or more recombinant DNAs, said recombinant DNAs comprising:

- a DNA encoding an RNA, protein or polypeptide, which when expressed in a plant cell inhibits or reduces the expression of an endogenous sesquiterpenoid synthase in said cell, and
- a plant expressible promoter, whereby said DNA is in the same transcriptional unit and under the control of said plant-expressible promoter; and

(b) regenerating said plant from said plant cell.

13. The process of claim 12, wherein said sesquiterpenoid synthase is a germacrene A synthase.

14. The process of claim 13, wherein said DNA encodes a sense or anti-sense RNA capable of inhibiting or reducing the expression of said endogenous germacrene A synthase.

15. The process of claim 14, wherein said DNA comprises a sequence having at least 70% sequence similarity to the nucleotide sequence of SEQ ID NO 3 or SEQ ID NO 4, or the complementary strand thereof.

16. A process for producing a plant with increased insect resistance, said process comprising increasing or inducing the expression of a protein or polypeptide having germacrene A synthase activity in said plant.

17. The process of claim 16, comprising

(a) introducing into the genome of a plant cell or tissue a recombinant DNA comprising:

- a DNA encoding a protein or polypeptide having germacrene A synthase activity, and
- a plant expressible promoter; said DNA being in the same transcriptional unit and under the control of said plant-expressible promoter; and

(b) regenerating said plant from said plant cell or tissue

18. The process of claim 17, wherein said DNA encodes a protein or polypeptide having at least 70%, sequence similarity to the amino acid sequence of SEQ ID NO 7 or to the sequence of SEQ ID NO 8.

19. The process of claim 17, wherein said DNA comprises a sequence having at least 70% sequence similarity to the nucleotide sequence of SEQ ID NO 3 or SEQ ID NO 4.

20. The process of claim 17, wherein said DNA comprises the sequence of SEQ ID NO 3 or SEQ ID NO 4.

21. A recombinant polynucleic acid comprising

(a) a DNA encoding an RNA or protein, which when expressed in a cell of a plant either induces, increases or decreases the expression of a germacrene A synthase in said cell, and

(b) a plant expressible promoter; wherein said DNA is in the same transcriptional unit and under the control of said plant expressible promoter.

22. The recombinant polynucleic acid of claim 21, wherein said DNA encodes an anti-sense RNA, a ribozyme or a sense RNA, which when

expressed in a cell of a plant decreases the expression of an endogenous germacrene A synthase in said cell.

23. The recombinant polynucleic acid of claim 21, wherein said DNA has at least 70% sequence similarity to the nucleotide sequence of SEQ ID NO 3 or SEQ ID NO 4 or the complementary strand thereof.

24. The recombinant polynucleic acid of claim 23, wherein said DNA comprises the nucleotide sequence of SEQ ID NO 3 or SEQ ID NO 4, or part thereof.

25. The recombinant polynucleic acid of claim 21, wherein said DNA comprises
- a first nucleotide sequence having at least 70% sequence similarity to the complementary sequence of SEQ ID NO. 3 or SEQ ID NO 4, or part thereof, and
- a second nucleotide sequence having at least 70% sequence similarity to the sequence of SEQ ID NO 3 or SEQ ID NO 4, or part thereof, and optionally,
- a spacer sequence between said first and said second sequence.

26. The recombinant polynucleic acid of claim 25, wherein said first nucleotide sequence is complementary to part of said second sequence.

27. The recombinant polynucleic acid of claim 21, wherein said DNA comprises
- a first nucleotide sequence having at least 70% sequence similarity to the sequence of SEQ ID NO 3 or SEQ ID NO 4, or part thereof, and
- a second nucleotide sequence having at least 70% sequence similarity to the sequence of SEQ ID NO 3 or SEQ ID NO 4, or part thereof.

28. The recombinant polynucleic acid of claim 21, wherein said DNA encodes a protein or polypeptide with germacren A synthase activity.

29. The recombinant polynucleic acid of claim 28, wherein said DNA encodes a protein or polypeptide having at least 70% sequence similarity to the sequence of SEQ ID NO 7 or SEQ ID NO 8.

30. The recombinant polynucleic acid of any one of claims 21 to 29, wherein said plant-expressible promoter is the promoter of an endogenous germacrene A synthase gene.

31. The recombinant polynucleic acid of any one of claims 21 to 29, wherein said plant-expressible promoter is a constitutive promoter.

32. The recombinant polynucleic acid of any one of claims 21 to 29, wherein said plant-expressible promoter is an inducible or a tissue-specific promoter.

33. A cell of a plant, transformed with the recombinant polynucleic acid of any one of claims 21 to 32.

34. A plant consisting essentially of the plant cells of claim 33.

35. The plant of claim 34, which is selected from the group of the genera Carum, Chichorium, Daucus, Juniperus, Chamomilla, Lactuca, Pogstemon, and Vetivera.

36. The seed of a plant of claim 35, comprising said recombinant DNA.

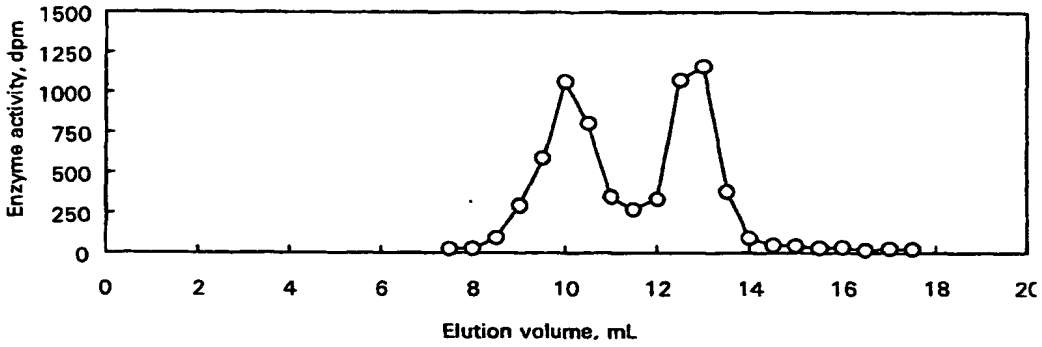
37. A probe which is part of a polynucleic acid sequence according to any of claims 1-9 and which hybridizes specifically with said polynucleic acid or the complement thereof.

38. A primer derived from a polynucleic acid sequence according to any of claims 1-9 and which specifically amplifies with said polynucleic acid or the complement thereof.

Abstract

This invention relates to the use of sesquiterpenoid synthase genes, particularly genes encoding germacrene A synthase, to modulate (i.e. repress, induce or increase) the expression or activity of sesquiterpenoid synthases in plants, so as to directly or indirectly influence taste, the production of sesquiterpene lactones, and/or resistance against insects, nematodes, micro-organisms and vertebrate herbivores in the plant.

Figure 1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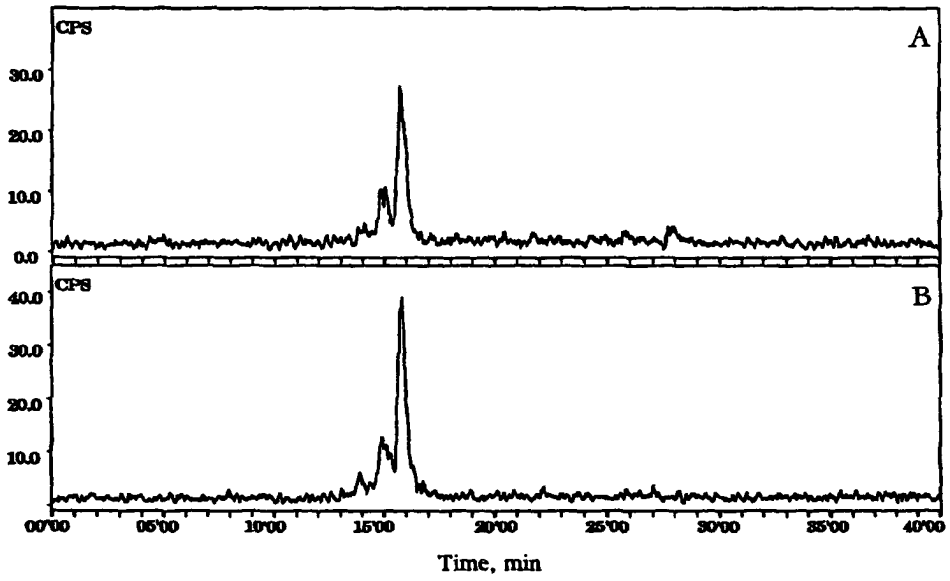
1000
750
500
250
0
0
2
4
6
8
10
12
14
16
18
20
Elution volume, mL

Figure 2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20.0
10.0
0.0
CPS
B
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30.0
20.0
10.0
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05'00
10'00
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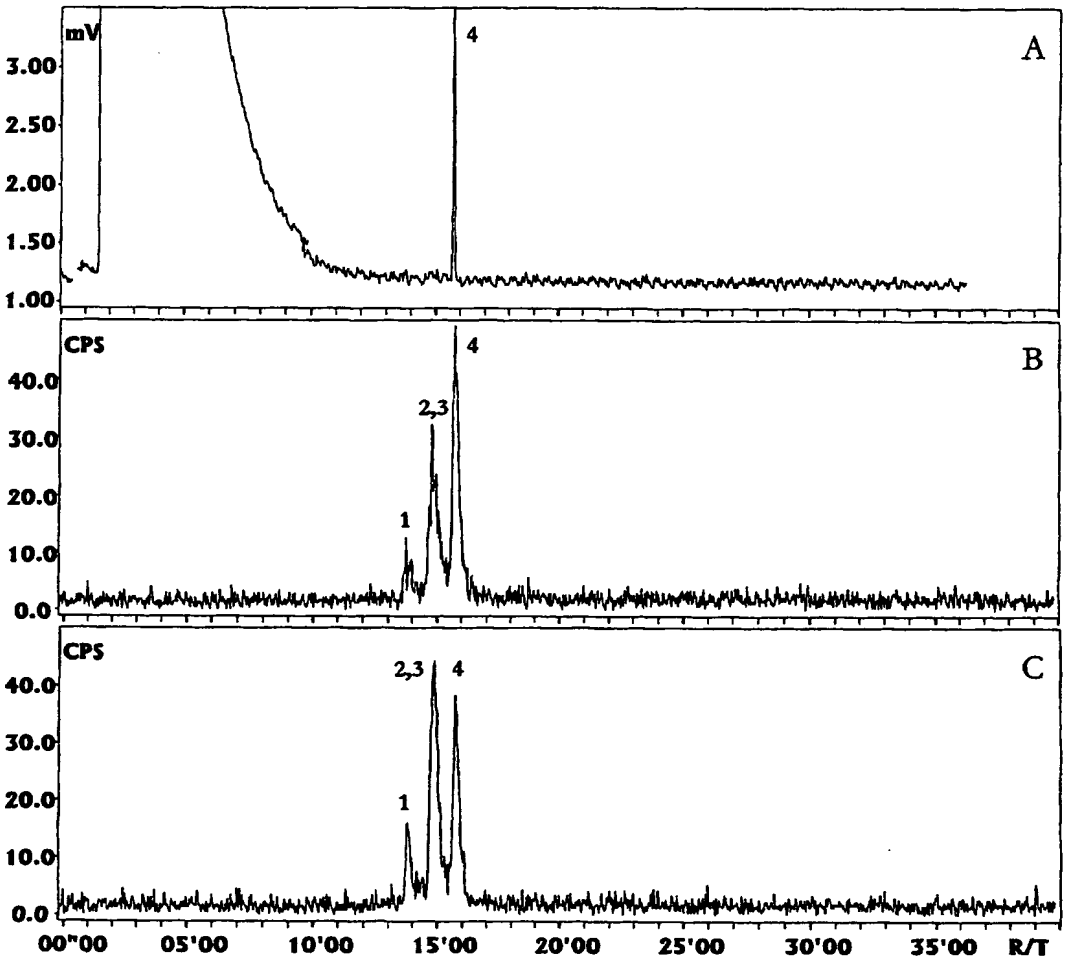
20'00
25'00
30'00
35'00
40'00
Time, min

Figure 3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2.00
1.50
1.00
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20.0
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0.0
2,3
1
4
B
CPS
40.0
30.0
20.0
10.0
0.0
2,3
4
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C
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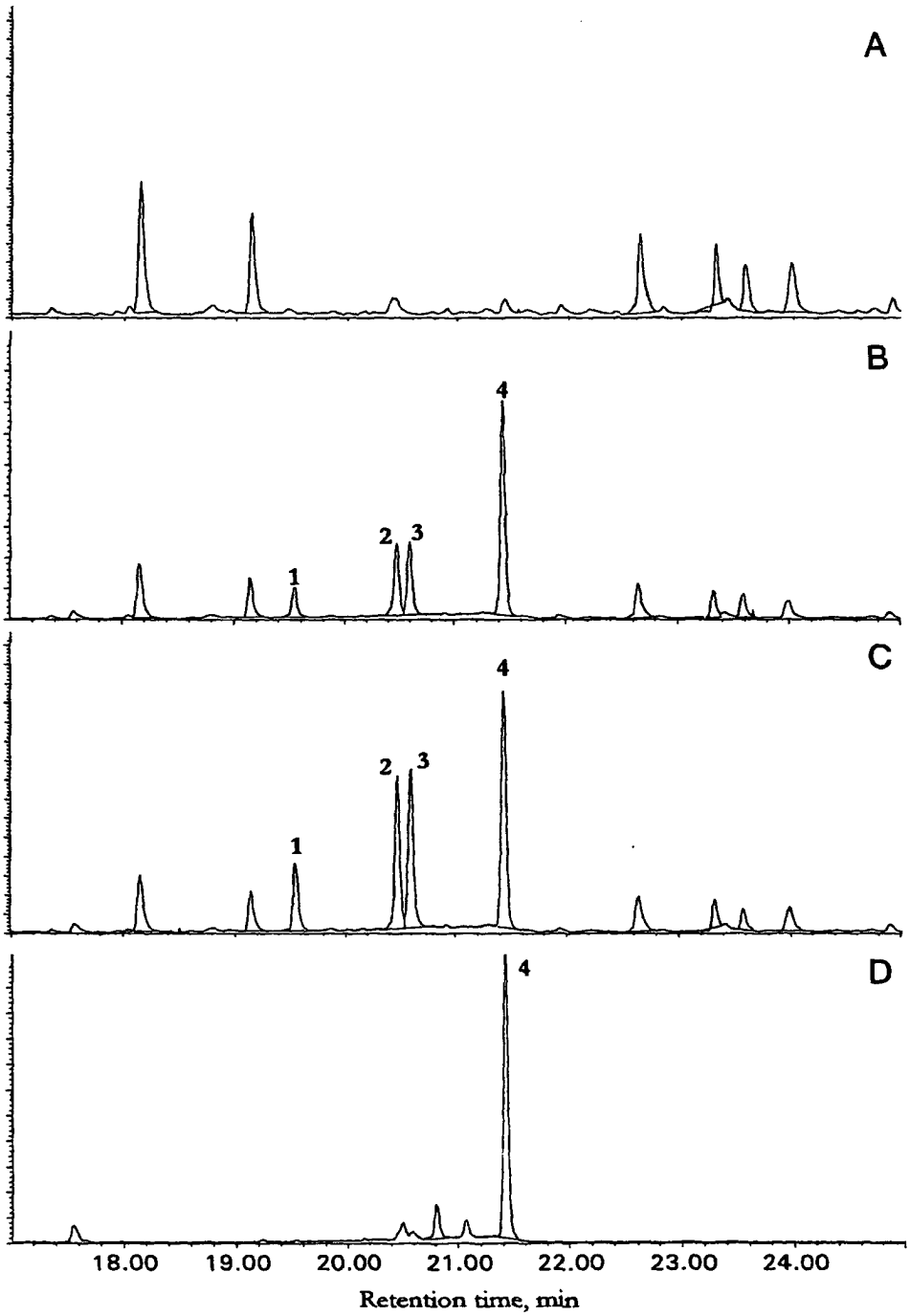
25'00
30'00
35'00
R/T

Figure 4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B
C
D
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2
3
4
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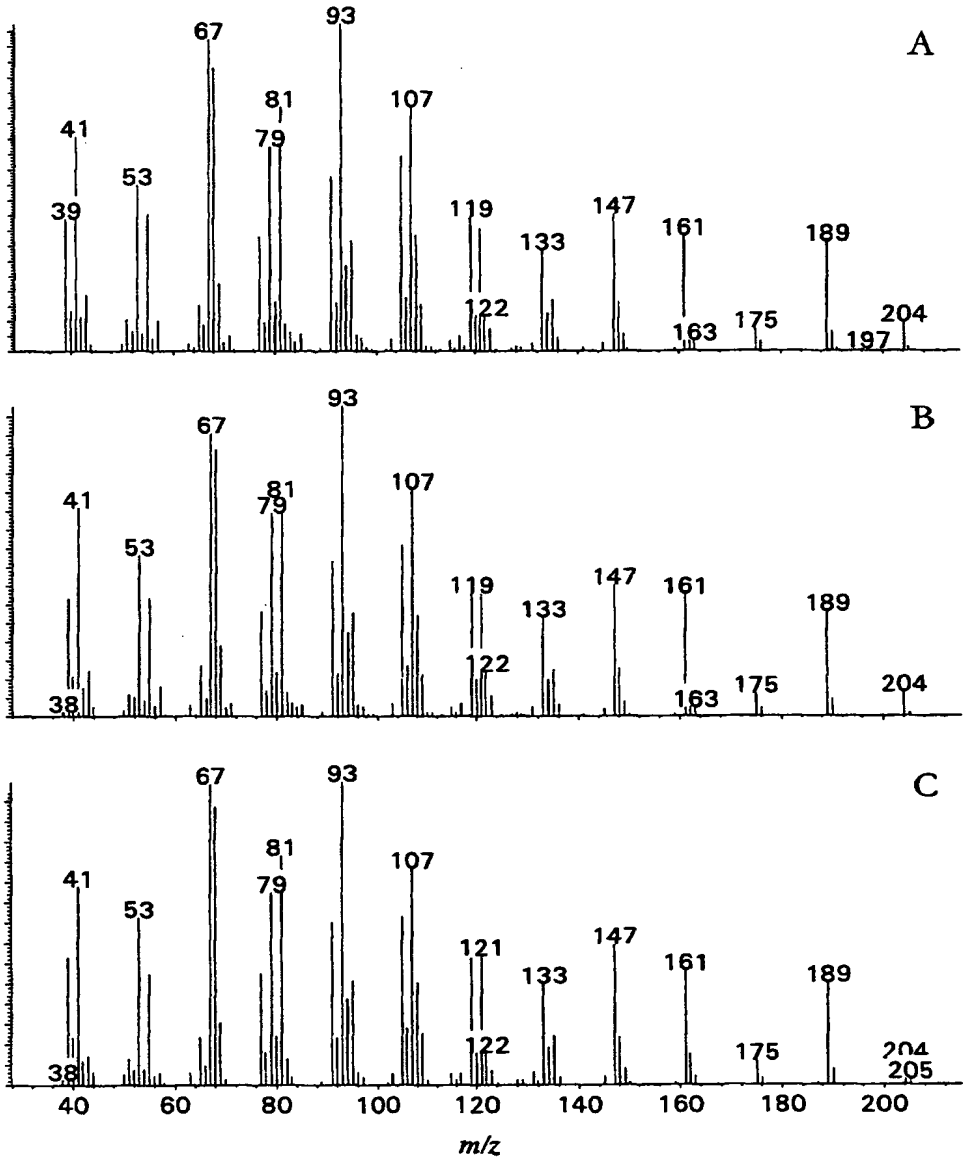
19.00
20.00
21.00
22.00
23.00
24.00
Retention time, min

Figure 5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B
C
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93
81
107
41
79
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39
119
147
161
133
189
122
175
204
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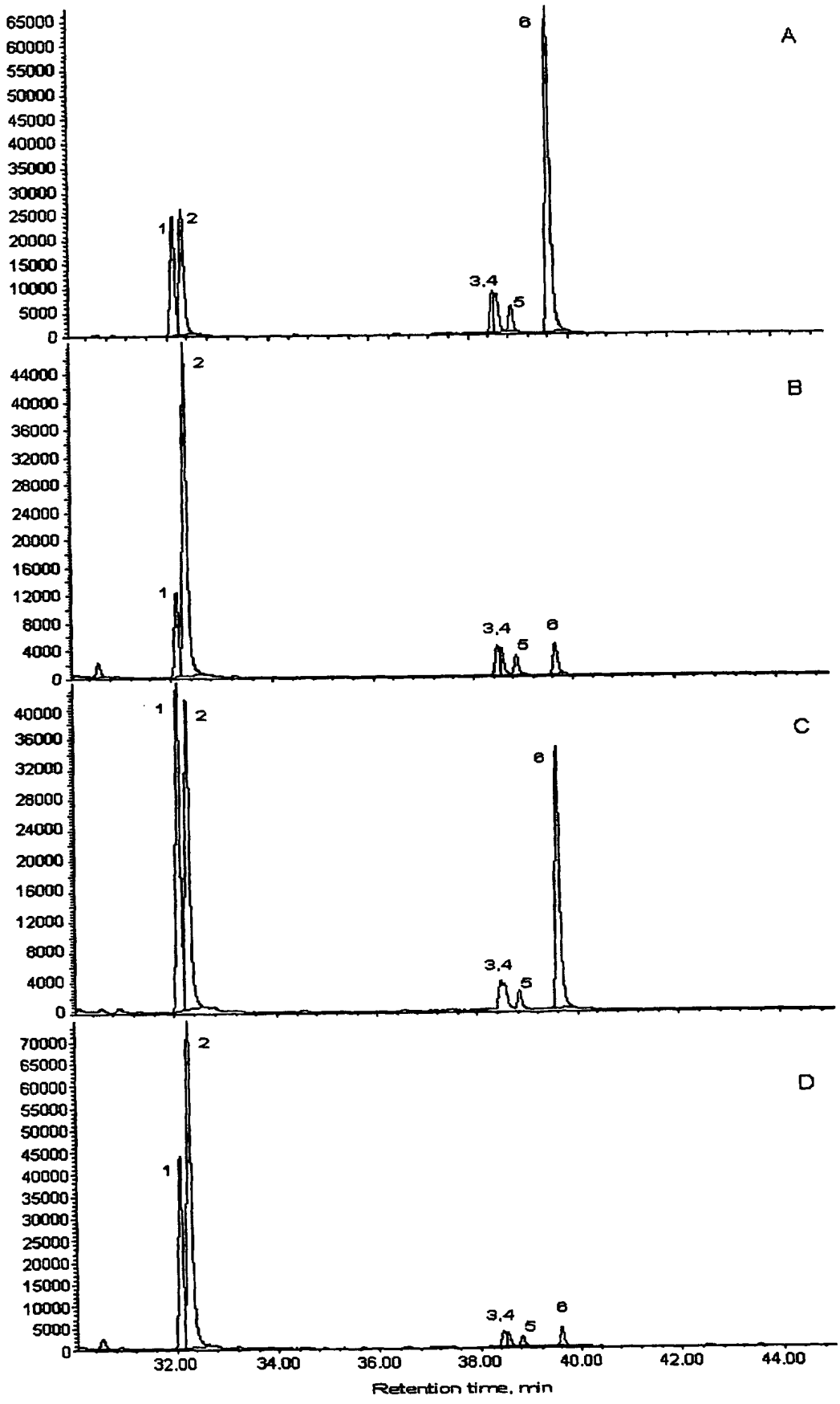
197
38
121
205
40
60
80
100
120
140
160
180
200
m/z

Figure 6


A
B
C
D
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2
3,4
5
6
Retention time, min
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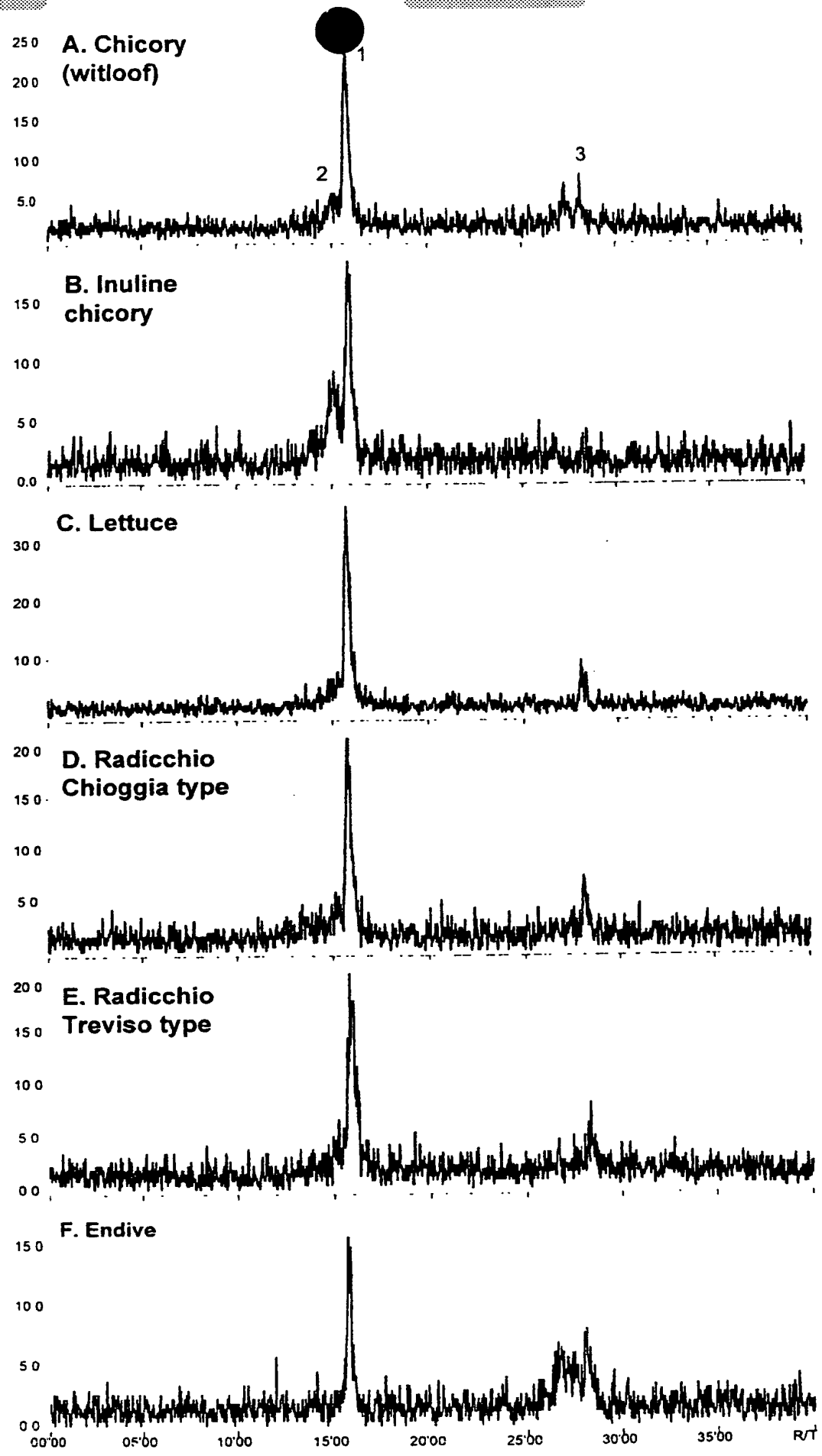
34.00
36.00
38.00
40.00
42.00
44.00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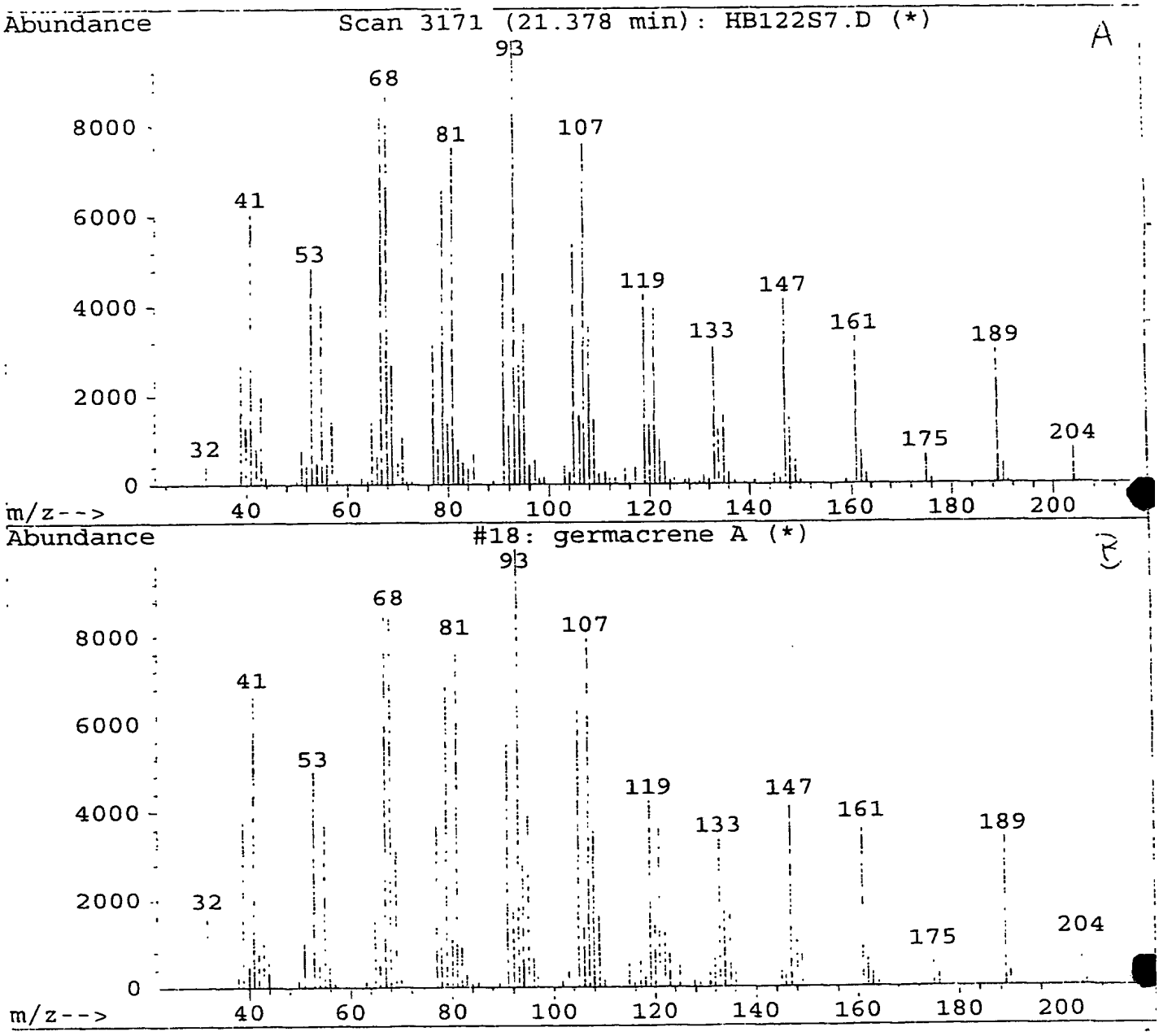
1
2
3
B. Inuline chicory
C. Lettuce
D. Radicchio Chioggia type
E. Radicchio Treviso type
F. Endive
00'00
05'00
10'00
15'00
20'00
25'00
30'00
35'00
R/T

Figure 7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93
68
81
107
41
53
119
147
133
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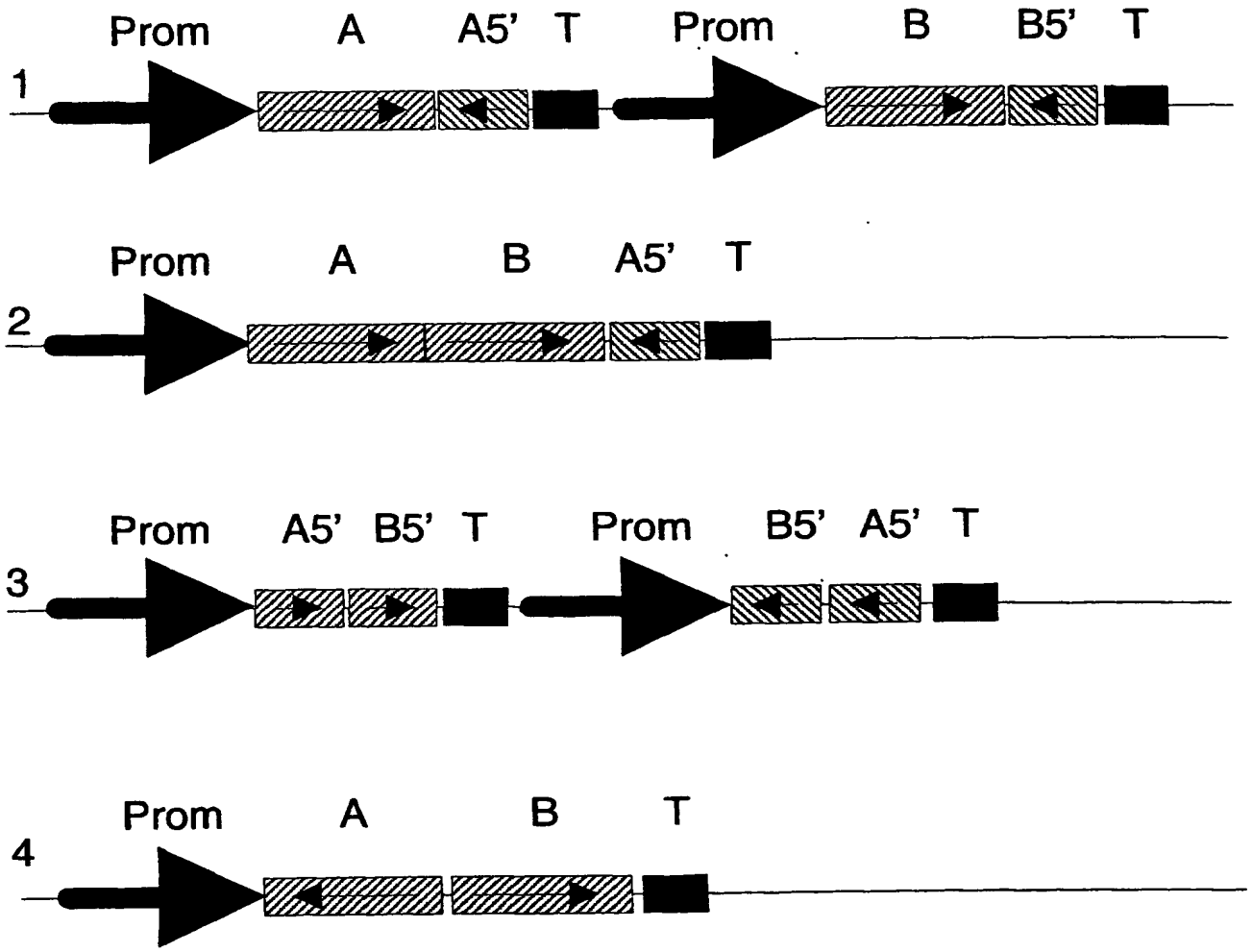
189
175
204
32
8000
6000
4000
2000
0
m/z-->
40
60
80
100
120
140
160
180
200
Abundance
#18: germacrene A (*)
R
93
68
81
107
41
53
119
147
133
161
189
175
204
32
8000
6000
4000
2000
0
m/z-->
40
60
80
100
120
140
160
180
200

Figure 9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3
Prom
A
B
T
4

Figure 10

```
A   -----ATCAAACTT-AGCTATATCCTT--------TCACCTT-----CAAGA-----AAT    36
B   GTTTCATTAAAGATCATCCA-ATCCGAAAAATGGCTCTCGTTAGAAACAACAGCAGCAAT    59
          ** ***  * * * * ****           ** * **     *** *     ***

A   GGCAG-----CAGTT----------GAAGCCA-ACGGTACCTTCCAAGC--AAACA----    74
B   GGTCGGGAGCCAGTTCTCAGCCCCAGAAGTCTCACAAGTCCT-CGAGGCTTAACCAGCCC   118
    **  *     *****           **** *  **    *** * * **  ** **

A   ---------------CCAAA--ACCACAGAGCCGGTGCGTCCTTTAGCCAACTTCCCTCC   117
B   CCGACCGTTGTCTGTCCAACCGACTCCAGAGCCGGTTCGACCTTTGGCCAACTTCCCACC   178
                    ****   **  ********** ** ***** ********** **

A   TTCTGTATGGGGTGATCGATTCTTGTCATTCTCTCTTGACACTACGGAATTGGAAGGATA   177
B   TTCGATCTGGGCTGATCGCTTCATCTCATTCTCTCTTGATAACTCTCAATTGGAAGCTTA   238
    ***  * **** ****** *** * ************ *   *  *********  **

A   TGCAAAAGCTATGGAGGAGCCAAAAGAAGAAGTGAGAAAACTGATCGTAGATCCAACAAT   237
B   TGCAAATGCACTTGAAGAACCAAAAGAAGCAGTGAAGAGTTTAATAACCGACACTACCAT   298
    ****** **  * ** ** ********** *****  *  * **    **  * ** **

A   GGATTCAAATAAGAAACTAAGTTTGATTTATTCTGTACACCGTCTTGGTTTGACATATCT   297
B   TGATGCAAACACAAAACTGAAATTGATTTATTCAGTGCACCGTCTTGGTTTGTCGTATCT   358
     *** **** *  ***** *  ********** ** ************** * *****

A   GT-TCTTGCAAGAGATTGAAGCGCAGCTTGACAAACTATTTAAAGAGTTTAACTTGCAAG   356
B   TTATCCAG-ATGAGATTGATGCCGAACTCAACAAACTCTTCGAGAAGATTGACTTACAGT   417
     * **  * * ******** **  * **  ******* **  *  ** ** **** **

A   ATTATGATGAGTTTGATCTATACACAACTTCTATTAACTTTCAAGTTTTCAGACACCTTG   416
B   ATTACGAACAAGTTGATTTGTACACTATTGCAGTACAATTTCAAGTTTTCAGACACCATG   477
    **** **  *  ***** * ***** * * *  *  * ******************** **
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A   GTCACAAACTGCCATGTGATGTGTTTAACAAATTCAAGGACAGTAGCTCGGGTACATTCA   476
B   GTTATAAAATTTCTTCTGATGTGTTTAAAAAGTTCAAGGACAGTACTACGGGTACATTCA   537
    ** * *** *  * * ************ ** ************   ***********

A   AGGAATCCATTACCAACGATGTGAAGGGTATGTTAGGCTTATATGAATCTGCACAATTGA   536
B   CGGATGATGTAACAAAAGACGTGAAGGGTATGCTGAGTTTATACGAATCAGCACACTTAA   597
     ***     * ** ** ** ************ *  * ***** ***** ***** ** *

A   GATTAAGAGGAGAACCCATTCTCGATGAAGCTTCCGCATTCACTGAAACTCAACTCAAGA   596
B   GGCTACACGGTGAAGATATCTTAGATGAAGCTTTAGCATTCACCGAAGCTCACCTTAAAA   657
    *  **   ** ***   **  * **********  ******** *** **** ** ** *

A   GTGTAGTAAACACTCTCGAAGGCAATCTTGCAAAACAGGTGATGCAATCATTGAGGAGAC   656
B   AAATCCTAACCACACTTGAAGGAGATCTTGCACGCCAAGTGAACCAAGTCTTAAAAAGAC   717
       *  *** *** ** *****  ********   ** ****  ***   ** *  ****

A   CATTCCATCAGGGGATGCCAATGGTTGAGGCAAGGATGTATTTCTCCAACTATGATGAAG   716
B   CCTTCCACACTGGAATGCCAATGGTAGAGGCACGGCTATATTTTATCACACACGAAGAAG   777
    * *****    ** ********** ****** ** * *****   **   * ** ****

A   AATGTTCCACACACGAGTCATTACCTAAGCTTGCAAAGCTTCATTTCAACTATTTGCAGC   776
B   ATTTTTCAAGCCATGAGTCGGTTGTAAAGCTAGCTAAAGTCCACTTCAACTATTTGCAAC   837
    * * *** *  ** *****  *    ***** ** **  * ** ************** *

A   TACAACAAAAGGAAGAACTTCGCATTGTCTCAAAGTGGTGGAAGGATATGAGGTTCCAGG   836
B   TACAACAAAAAGAAGAACTACGACTCGTGTCACAGTGGTGGAAAGATATGCAATTCCAAC   897
    ********** ******** **  * ** *** ********** ******   *****

A   AAACTACTCCTTACATAAGGGATAGAGTACCAGAGATTTACTTATGGATATTGGGATTGT   896
B   AATCCGTCCCTTACATAAGAGATAGAGTACCGGAGATATACCTATGGATTTTGGGGTTAT   957
    ** *    ********** ********** ***** *** ******* ***** ** *
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A  ACTTTGAGCCTCGTTACTCCTTGGCACGAATCATCGCCACAAAATTACATTGTTCCTCG   956
B  ATTTCGAGCCGTATTACTCTCGGGCACGTATCATAGCCACTAAAATCACGTTGTTCTTGG 1017
   * ** *****   ******   ****** ***** ***** ***** ** ****** * *

A  TGGTTCTAGATGACACATATGATGCATACGCTACCATTGAAGAGATTCGACTTCTGACTG 1016
B  TGGTTTTGGACGATACATATGACGCGTATGCTACAATTGACGAGATCCGATCGATCACAG 1077
   ***** * ** ** ******** ** ** ***** ***** ***** ***     * ** *

A  ATGCCATAAACAGGTGGGACATCAGTGCTATGGAGCAAATTCCGGAATATATTCGACCAT 1076
B  ATGCGATTAATAGGTGGGAAATTAGCGCGATCGACCAACTTCCTGAATATATCAAACCGT 1137
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A  TCTACAAAATTCTCCTAGATGAGTATGCTGAACTTGAGAAGCAACTAGCTAAAGAAGGAA 1136
B  TCTACAGAATTCTCCTCAACGAATATGATGACCTCGAGAAAGAATACTCAAAGGACGGAA 1197
   ****** *********  * ** **** *** ** *****  **     * ** ** ****

A  GAGCAAAAAGTGTTATTGCTTCAAAAGAAGCGTTCCAGGACATTGCAAGAGGATACCTTG 1196
B  GAGCGTTCAGTGTCCACGCTTCAAAACAAGCGTTTCAAGAAATCGCGAGAGGGTATCTTG 1257
   ****     *****    ********* ******* ** ** ** ** ***** ** ****

A  AAGAGGCCGAGTGGACAAACAGTGGATACGTGGCATCATTTCCAGAGTATATGAAGAACG 1256
B  AAGAGGCGGAGTGGTTACACAACGGTTATGTGGCAACATTTCCCGAGTATATGAAGAATG 1317
   ******* ******  * ***   ** ** ****** ******* ************* *

A  GTTTAATTACTTCTGCTTACAATGTTATTTCAAAATCTGCTTTAGTGGGTATGGGCGAGA 1316
B  GTTTGATTACTTCGGCTTATAATGTCATTTCAAAATCCGCATTGGTGGGAATGGGTGCGA 1377
   **** ******** ***** ***** *********** ** ** ***** ***** * **

A  TGGTTGGTGAAGATGCCTTGGCTTGGTATGAAAGTCATCCAAAGACATTGCAAGCTTCAG 1376
B  TTGCAGATGAAGAGGCTCTTGCTTGGTATGAAACACATCCGAAAATTTTGAAAGCTTCAG 1437
   * *  * ****** **  * ************  ***** ** *  *** *********

A  AGTTAATTTCAAGACTCCAAGATGATGTGATGACTTACCAGTTTGAGCGAGAAAGGGGAC 1436
B  AGTTGATTTCAAGGCTCCAAGACGATGTTATGACTTTCCAGTTTGAGAGAAAACGAGGAC 1497
   **** ******** ******** ***** ******* ********** ** ** * ****
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```
A  AATCAGCCACCGGCGTTGATTCTTATATCAAGACCTATGGCGTAACAGAAAAGGAAGCGA 1496
B  AATCAGCAACTGGTGTGGATGCTTATATCAAGGAATACAATGTATCCGAAGAAGTAGCGA 1557
   ******* ** ** ** *** **********    **     *** * *** * * *****

A  TTGACGAGCTAAACAAAATGATTGAAAATGCATGGAAGGATATAAATGAGGGCTGCCTTA 1556
B  TCAAAGAGCTCATGAAGATGATTGAAAACGCATGGAAAGATATAAATGAGGGATGCTTGA 1617
   *  * ***** *  ** ********** ******** ************** *** * *

A  AGCCAAGAGAAGTCTCAATGGATTTGCTTGCCCCAATTCTTAATCTTGCACGAATGATAG 1616
B  AGCCCACTGAGGTCTCGGTGGCTCTACTAACTCCTATTTTGAATCTCGCGAGAATGATAG 1677
   **** *  ** *****  *** * * **  * ** *** * ***** **  *********

A  ATGTGGTATACAGGTACGACGATGGGTTCACTTTTCCGGGAAAGACCATGAAAGAGTATA 1676
B  ATGTCGTATACAAATTCGATGATGGATTCACCTTTCCCGGGAAAACCCTAAAAGACTATA 1737
   **** *******  * *** ***** ***** ***** ** ** *** * ***** ****

A  TTACTCTGTTGTTTGTTGGTTCTTCACCCA-------------------TGTAATA---- 1713
B  TTACCCTTTTGTTCGTTAGTCCTCCACCGAGTCTCGAAAACTGATAGGTTGTAATATAAG 1797
   **** ** ***** *** ** ** **** *                    *******

A  ----ATA----------ATTTT--TATTTCAT-------ATGTTT--------------- 1735
B  CATCATATTTTCTCGGAATTTTGCTTTTTCATGTTGCGTATGTTTGCGACTTTCGCTCAA 1857
       ***           *****  * ******       ******

A  ------------------------------------------------------------
B  TTTGGTGAGATTCTTTTTGACATTCTATTGTATAATCTTCTTTTGTTCAAATAAAAAGTT 1917

A  --------------AAAAAAAAAAAAAAAAAAA-- 1755
B  TCTTCTTATGGACTAAAAAAAAAAAAAAAAAAAAA 1953
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```

Figure 11

```
A   MALVRNNSSNGREPVLSPRSLTSPRGLTSPRPLSVQPTPEPVRPLANFPPSIWADRFISF  60
B   MAAVEAN------------------GTFQAN----TKTTEPVRPLANFPPSVWGDRFLSF  38
    ** *. *                  *  ...      *.***********:*.***:**

A   SLDNSQLEAYANALEEPKEAVKSLITDTTIDANTKLKLIYSVHRLGLSYLYPDEIDAELN 120
B   SLDTTELEGYAKAMEEPKEEVRKLIVDPTMDSNKKLSLIYSVHRLGLTYLFLQEIEAQLD  98
    ***.::**.**:*:***** *:.**.*.*:*:*.**.**********:**: :**:*:*:

A   KLFEKIDLQYYEQVDLYTIAVQFQVFRHHGYKISSDVFKKFKDSTTGTFTDDVTKDVKGM 180
B   KLFKEFNLQDYDEFDLYTTSINFQVFRHLGHKLPCDVFNKFKDSSSGTFKESITNDVKGM 158
    ***:::**  *::.**** :::****** *:*:..***:*****::***.:.:*:*****

A   LSLYESAHLRLHGEDILDEALAFTEAHLKKILTTLEGDLARQVNQVLKRPFHTGMPMVEA 240
B   LGLYESAQLRLRGEPILDEASAFTETQLKSVVNTLEGNLAKQVMQSLRRPFHQGMPMVEA 218
    *.*****:***:** ***** ****::**.::.****:**:** * *:**** *******

A   RLYFITHEEDFSSHESVVKLAKVHFNYLQLQQKEELRLVSQWWKDMQFQQSVPYIRDRVP 300
B   RMYFSNYDEECSTHESLPKLAKLHFNYLQLQQKEELRIVSKWWKDMRFQETTPYIRDRVP 278
    *:** .::*: *:***: ****:**************:**:*****:**::.********

A   EIYLWILGLYFEPYYSRARIIATKITLFLVVLDDTYDAYATIDEIRSITDAINRWEISAI 360
B   EIYLWILGLYFEPRYSLARIIATKITLFLVVLDDTYDAYATIEEIRLLTDAINRWDISAM 338
    ************* ** *************************:*** :*******:***:

A   DQLPEYIKPFYRILLNEYDDLEKEYSKDGRAFSVHASKQAFQEIARGYLEEAEWLHNGYV 420
B   EQIPEYIRPFYKILLDEYAELEKQLAKEGRAKSVIASKEAFQDIARGYLEEAEWTNSGYV 398
    :*:****:***:***:** :***: :*:*** ** ***:***:********** :.***
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B   ASFPEYMKNGLITSAYNVISKSALVGMGEMVGEDALAWYESHPKTLQASELISRLQDDVM 458
    *:************************** :..*:******:*** *:*************

A   TFQFERKRGQSATGVDAYIKEYNVSEEVAIKELMKMIENAWKDINEGCLKPTEVSVALLT 540
B   TYQFERERGQSATGVDSYIKTYGVTEKEAIDELNKMIENAWKDINEGCLKPREVSMDLLA 518
    *:****:*********:*** *.*:*:* **.** ***************** ***: **:

A   PILNLARMIDVVYKFDDGFTFPGKTLKDYITLLFVSPPPSLEN 583
B   PILNLARMIDVVYRYDDGFTFPGKTMKEYITLLFVGSSPM--- 558
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<120> Use of Sesquiterpenoid synthase genes to influence bitterness and resistance in plants

<130> EP.99.AB-DLO.GERM

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Ile Leu Asp Glu Ala Ser Ala Phe Thr Glu Thr Gln Leu Lys Ser Val
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Val Asn Thr Leu Glu Gly Asn Leu Ala Lys Gln Val Met Gln Ser Leu
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Arg Arg Pro Phe His Gln Gly Met Pro Met Val Glu Ala Arg Met Tyr
65                  70                  75                  80

Phe Ser Asn Tyr Asp Glu Glu Cys Ser Thr His Glu Ser Leu Pro Lys
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Leu Ala Lys Leu His Phe Asn Tyr Leu Gln Leu Gln Gln Lys Glu Glu
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Leu Arg Ile Val Ser Lys Trp Trp Lys Asp Met Arg Phe Gln Glu Thr
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Gly Leu Tyr Phe Glu Pro Arg Tyr Ser Trp Ala Arg Ile Ile Ala Thr
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35 40 45

Thr Glu Ala His Leu Lys Lys Ile Leu Thr Thr Leu Glu Gly Asp Leu
50 55 60

Ala Arg Gln Val Asn Gln Val Leu Lys Arg Pro Phe His Thr Gly Met
65 70 75 80

Pro Met Val Glu Ala Arg Leu Tyr Phe Ile Thr His Glu Glu Asp Phe
85 90 95

Ser Ser His Glu Ser Val Val Lys Leu Ala Lys Val His Phe Asn Tyr
100 105 110

Leu Gln Leu Gln Gln Lys Glu Glu Leu Arg Leu Val Ser Gln Trp Trp
115 120 125

Lys Asp Met Gln Phe Gln Gln Ser Val Pro Tyr Ile Arg Asp Arg Val
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Asp Pro Thr Met Asp Ser Asn Lys Lys Leu Ser Leu Ile Tyr Ser Val
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His Lys Leu Pro Cys Asp Val Phe Asn Lys Phe Lys Asp Ser Ser Ser
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Gly Thr Phe Lys Glu Ser Ile Thr Asn Asp Val Lys Gly Met Leu Gly
145 150 155 160

Leu Tyr Glu Ser Ala Gln Leu Arg Leu Arg Gly Glu Pro Ile Leu Asp
165 170 175

Glu Ala Ser Ala Phe Thr Glu Thr Gln Leu Lys Ser Val Val Asn Thr
180 185 190

Leu Glu Gly Asn Leu Ala Lys Gln Val Met Gln Ser Leu Arg Arg Pro
195 200 205

Phe His Gln Gly Met Pro Met Val Glu Ala Arg Met Tyr Phe Ser Asn
210 215 220

Tyr Asp Glu Glu Cys Ser Thr His Glu Ser Leu Pro Lys Leu Ala Lys
225 230 235 240

Leu His Phe Asn Tyr Leu Gln Leu Gln Gln Lys Glu Glu Leu Arg Ile
245 250 255

Val Ser Lys Trp Trp Lys Asp Met Arg Phe Gln Glu Thr Thr Pro Tyr

260 265 270

Ile Arg Asp Arg Val Pro Glu Ile Tyr Leu Trp Ile Leu Gly Leu Tyr
275 280 285

Phe Glu Pro Arg Tyr Ser Leu Ala Arg Ile Ile Ala Thr Lys Ile Thr
290 295 300

Leu Phe Leu Val Val Leu Asp Asp Thr Tyr Asp Ala Tyr Ala Thr Ile
305 310 315 320

Glu Glu Ile Arg Leu Leu Thr Asp Ala Ile Asn Arg Trp Asp Ile Ser
325 330 335

Ala Met Glu Gln Ile Pro Glu Tyr Ile Arg Pro Phe Tyr Lys Ile Leu
340 345 350

Leu Asp Glu Tyr Ala Glu Leu Glu Lys Gln Leu Ala Lys Glu Gly Arg
355 360 365

Ala Lys Ser Val Ile Ala Ser Lys Glu Ala Phe Gln Asp Ile Ala Arg
370 375 380

Gly Tyr Leu Glu Glu Ala Glu Trp Thr Asn Ser Gly Tyr Val Ala Ser
385 390 395 400

Phe Pro Glu Tyr Met Lys Asn Gly Leu Ile Thr Ser Ala Tyr Asn Val
405 410 415

Ile Ser Lys Ser Ala Leu Val Gly Met Gly Glu Met Val Gly Glu Asp
420 425 430

Ala Leu Ala Trp Tyr Glu Ser His Pro Lys Thr Leu Gln Ala Ser Glu
435 440 445

Leu Ile Ser Arg Leu Gln Asp Asp Val Met Thr Tyr Gln Phe Glu Arg
450 455 460

Glu Arg Gly Gln Ser Ala Thr Gly Val Asp Ser Tyr Ile Lys Thr Tyr
465 470 475 480

Gly Val Thr Glu Lys Glu Ala Ile Asp Glu Leu Asn Lys Met Ile Glu
485 490 495

Asn Ala Trp Lys Asp Ile Asn Glu Gly Cys Leu Lys Pro Arg Glu Val
500 505 510

Ser Met Asp Leu Leu Ala Pro Ile Leu Asn Leu Ala Arg Met Ile Asp

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        515                 520                 525

Val Val Tyr Arg Tyr Asp Asp Gly Phe Thr Phe Pro Gly Lys Thr Met
    530                 535                 540

Lys Glu Tyr Ile Thr Leu Leu Phe Val Gly Ser Ser Pro Met
545                 550                 555


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<213> Cichorium intybus

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Met Ala Leu Val Arg Asn Asn Ser Ser Asn Gly Arg Glu Pro Val Leu
  1               5                  10                  15

Ser Pro Arg Ser Leu Thr Ser Pro Arg Gly Leu Thr Ser Pro Arg Pro
             20                  25                  30

Leu Ser Val Gln Pro Thr Pro Glu Pro Val Arg Pro Leu Ala Asn Phe
         35                  40                  45

Pro Pro Ser Ile Trp Ala Asp Arg Phe Ile Ser Phe Ser Leu Asp Asn
     50                  55                  60

Ser Gln Leu Glu Ala Tyr Ala Asn Ala Leu Glu Glu Pro Lys Glu Ala
 65                  70                  75                  80

Val Lys Ser Leu Ile Thr Asp Thr Thr Ile Asp Ala Asn Thr Lys Leu
                 85                  90                  95

Lys Leu Ile Tyr Ser Val His Arg Leu Gly Leu Ser Tyr Leu Tyr Pro
            100                 105                 110

Asp Glu Ile Asp Ala Glu Leu Asn Lys Leu Phe Glu Lys Ile Asp Leu
        115                 120                 125

Gln Tyr Tyr Glu Gln Val Asp Leu Tyr Thr Ile Ala Val Gln Phe Gln
    130                 135                 140

Val Phe Arg His His Gly Tyr Lys Ile Ser Ser Asp Val Phe Lys Lys
145                 150                 155                 160

Phe Lys Asp Ser Thr Thr Gly Thr Phe Thr Asp Asp Val Thr Lys Asp
                165                 170                 175
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Val Lys Gly Met Leu Ser Leu Tyr Glu Ser Ala His Leu Arg Leu His
180 185 190

Gly Glu Asp Ile Leu Asp Glu Ala Leu Ala Phe Thr Glu Ala His Leu
195 200 205

Lys Lys Ile Leu Thr Thr Leu Glu Gly Asp Leu Ala Arg Gln Val Asn
210 215 220

Gln Val Leu Lys Arg Pro Phe His Thr Gly Met Pro Met Val Glu Ala
225 230 235 240

Arg Leu Tyr Phe Ile Thr His Glu Glu Asp Phe Ser Ser His Glu Ser
245 250 255

Val Val Lys Leu Ala Lys Val His Phe Asn Tyr Leu Gln Leu Gln Gln
260 265 270

Lys Glu Glu Leu Arg Leu Val Ser Gln Trp Trp Lys Asp Met Gln Phe
275 280 285

Gln Gln Ser Val Pro Tyr Ile Arg Asp Arg Val Pro Glu Ile Tyr Leu
290 295 300

Trp Ile Leu Gly Leu Tyr Phe Glu Pro Tyr Tyr Ser Arg Ala Arg Ile
305 310 315 320

Ile Ala Thr Lys Ile Thr Leu Phe Leu Val Val Leu Asp Asp Thr Tyr
325 330 335

Asp Ala Tyr Ala Thr Ile Asp Glu Ile Arg Ser Ile Thr Asp Ala Ile
340 345 350

Asn Arg Trp Glu Ile Ser Ala Ile Asp Gln Leu Pro Glu Tyr Ile Lys
355 360 365

Pro Phe Tyr Arg Ile Leu Leu Asn Glu Tyr Asp Asp Leu Glu Lys Glu
370 375 380

Tyr Ser Lys Asp Gly Arg Ala Phe Ser Val His Ala Ser Lys Gln Ala
385 390 395 400

Phe Gln Glu Ile Ala Arg Gly Tyr Leu Glu Glu Ala Glu Trp Leu His
405 410 415

Asn Gly Tyr Val Ala Thr Phe Pro Glu Tyr Met Lys Asn Gly Leu Ile
420 425 430

Thr Ser Ala Tyr Asn Val Ile Ser Lys Ser Ala Leu Val Gly Met Gly
435 440 445

Ala Ile Ala Asp Glu Glu Ala Leu Ala Trp Tyr Glu Thr His Pro Lys
450 455 460

Ile Leu Lys Ala Ser Glu Leu Ile Ser Arg Leu Gln Asp Asp Val Met
465 470 475 480

Thr Phe Gln Phe Glu Arg Lys Arg Gly Gln Ser Ala Thr Gly Val Asp
485 490 495

Ala Tyr Ile Lys Glu Tyr Asn Val Ser Glu Glu Val Ala Ile Lys Glu
500 505 510

Leu Met Lys Met Ile Glu Asn Ala Trp Lys Asp Ile Asn Glu Gly Cys
515 520 525

Leu Lys Pro Thr Glu Val Ser Val Ala Leu Leu Thr Pro Ile Leu Asn
530 535 540

Leu Ala Arg Met Ile Asp Val Val Tyr Lys Phe Asp Asp Gly Phe Thr
545 550 555 560

Phe Pro Gly Lys Thr Leu Lys Asp Tyr Ile Thr Leu Leu Phe Val Ser
565 570 575

Pro Pro Pro Ser Leu Glu Asn
580

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<223> n = i

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ttycargayg araayggnaa rttyaarga 29

<210> 10

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<220>
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<223> n = i

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ccrtangcrt craangtrtc rtc 23

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<211> 23
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for A gene

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<210> 12
<211> 22
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primer for A gene

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<210> 13
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<212> DNA
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primer for B gene

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